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Role of Intracellular Chloride in the Reversible Activation of Neutrophil β_2 Integrins: A Lesson from TNF Stimulation[1]

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The process of β_2 integrin activation, which enhances the interaction of these heterodimers with ligands, plays a crucial role in the adherence-dependent neutrophilic polymorphonuclear leukocytes' (PMN) responses to TNF. Our previous observation, showing that a marked decrease of the high basal Cl^- content (Cl^-_i) is an essential step in the TNF-induced activation of PMN, stimulated this study, which investigates the role of alterations of Cl^-_i in the activation of β_2 integrins triggered by TNF. Here we show that TNF enhances the expression of activation-specific neoepitopes of β_2 integrins, namely, epitope 24, a unique epitope present on all three leukocyte integrin α subunits, and epitope CBRM1/5, localized to the I domain on the α-chain of Mac-1 (CD11bCD18). Moreover, we demonstrate that the conformational changes underlying the expression of the neoepitopes are dependent on a drop in Cl^-_i because 1) inhibition of Cl^-_i decrease is invariably accompanied by inhibition of β_2 integrin activation, 2) Cl^-_i decrease induced by means other than agonist stimulation, i.e., by placing PMN in Cl^--free buffers, activates β_2 integrins, and 3) restoration of the original Cl^-_i levels is accompanied by deactivation of β_2 integrins. We also show that Cl^-_i decrease is required for TNF-induced cytoplasmic alkalinization, but such a rise in pH_i does not seem to be relevant for β_2 integrin activation. The results of our study emphasize the role of Cl^- as a new PMN "second messenger." ***The Journal of Immunology*****, 2000, 165: 4606–4614.**

TNF may be regarded as an incomplete agonist of neutrophilic polymorphonuclear leukocytes (PMN)[3] because it is unable to activate the respiratory burst and degranulation of PMN in suspension, but it can do so when the cells are in contact with biologic surfaces, such as serum proteins or extracellular matrix components immobilized onto solid supports (1–10). Under these conditions, PMN exposed to TNF adhere to the substrate, spread on it, mount a vigorous respiratory burst, and release granule components. The whole phenomenon is mediated by the engagement of proadhesive molecules belonging to the β_2 integrin subfamily (CD11/CD18), namely, CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1, CR3), and CD11c/CD18 (gp150/95, CR4) (11). This is unequivocally proved by the finding that an inborn defect of CD18 expression, i.e., the type 1 leukocyte adhesion deficiency (LAD-1) syndrome (12), markedly impairs the TNF-induced activation of PMN in contact with biologic surfaces (1).

The mechanistic picture of this phenomenon, which is gradually emerging, is the following: 1) the engagement of the TNF receptors, particularly the 55-kDa receptor (TNFR-55) (7), delivers intracellular signals that up-regulate β_2 integrin functional activity, thereby promoting PMN adherence; 2) once adherent, the cells would rely on the signaling ability of β_2 integrins to trigger spreading and metabolic burst via a decrease in cAMP levels and protein tyrosine phosphorylation (5, 13). Indeed, several laboratories, including our own, have demonstrated a cooperation between signals generated by β_2 integrins and those originating from receptors for soluble agonists (5–9, 13–15). A new leap in the interpretation of PMN activation on biologic surfaces by TNF was made when the central role of a decrease in intracellular chloride content (Cl^-_i) in this phenomenon was recognized (9).

PMN use part of their energy to pump chloride ions from the extracellular environment into the intracellular compartment, thereby accumulating a considerable amount of this anion (16). Indeed, a distinct feature of resting PMN is an unusually high (80–100 mM) Cl^- concentration that is 4- to 5-fold higher than predicted on the basis of the Nernst equation (16, 17). The meaning of such an excess of Cl^-_i, as compared with other cells, is as yet unclear, although a Cl^- efflux seems to be one of the early PMN responses to several soluble agonists (18, 19), TNF being the most powerful in this respect (18).

The phenomenon of TNF-induced activation of PMN in contact with biologic surfaces is accompanied by a marked and long lasting Cl^- efflux and a corresponding decrease in Cl^-_i. Inhibition of Cl^- efflux and, hence, Cl^-_i decrease, prevents TNF-dependent activation (9). Because the phenomenon of PMN activation by TNF may encompass several steps (for example, TNF-TNF-Rs binding, β_2 integrin expression, β_2 integrin activation, cell adherence to the substrate, cytoskeleton reorganization, NADPH-oxidase assembly, etc.), one wonders which is (are) the crucial step(s) under Cl^-_i control.

This study investigates the mechanisms by which TNF regulates the functional activity of β_2 integrins. Here we show that 1) TNF induces the expression of Mg^{2+}-dependent, activation-specific neoepitopes, namely, epitope 24, a unique epitope present on all three leukocyte integrin α subunits (20), and epitope CBRM1/5, localized to the I domain on the α-chain of Mac-1 (21); 2) the

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[3] Abbreviations used in this paper: PMN, neutrophilic polymorphonuclear leukocytes; Cl^-_i, intracellular chloride content; pH_i, intracellular pH; FN, fibronectin; FBG, fibrinogen; MK447/A, 2-aminomethyl-4-(1-methyl-1-phenylethyl)-6-iodophenol hydrochloride; EA, [2,3-dichloro-4-(2-methylene-butyryl)phenoxy]acetic acid (etacrynic acid); DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; NHA, 5-*N*,*N*-hexamethylene amiloride; BCECF-AM, 2′,7′-bis-(2-carboxyethyl)-5,6-carboxyfluorescein acetoxymethyl ester.

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conformational changes underlying the expression of the aforementioned neoepitopes are dependent on a TNF-induced drop in Cl^-_i, because 1) inhibition of Cl^-_i decrease is invariably accompanied by inhibition of β_2 integrin activation, 2) Cl^-_i decrease induced by means other than agonist stimulation, i.e., by placing PMN in Cl^--free buffers, activates β_2 integrins, and 3) restoration of the original Cl^-_i levels is accompanied by deactivation of β_2 integrins.

TNF has been shown to induce in PMN in suspension a cytoplasmic alkalinization that is concomitant with Cl^- efflux (18). Thus, the question arises as to whether changes in intracellular pH (pH_i) and Cl^-_i are mutually dependent events and, more importantly, whether the rise in pH_i may have a role in the up-regulation of β_2 integrin functional activity. Here we show that the TNF-induced cytoplasmic alkalinization, which indeed accompanies the drop in Cl^-_i, is under the control of Cl^-_i changes, because inhibition of Cl^-_i decrease led to a marked impairment of cytoplasmic alkalinization. In contrast, inhibition of alkalinization had no effect on the drop in Cl^-_i. In addition, we show that a rise in pH_i does not seem to be required for β_2 integrin activation.

Materials and Methods

Reagents

Percoll was obtained from Pharmacia (Uppsala, Sweden). Fibronectin (FN) was purified from human plasma by affinity chromatography on gelatin as previously described (22). Human rTNF, produced in the yeast *Pichia pastoris*, was obtained from Bissendorf Biochemicals (Hannover, Germany). 2-Aminomethyl-4-(1-methyl-1-phenylethyl)-6-iodophenol hydrochloride (MK-447/A) was generously provided by Merck Sharp & Dohme Research Laboratories (Rahway, NJ). BSA, fibrinogen (FBG) fraction I from human plasma, [2,3-dichloro-4-(2-methylene-butyryl)phenoxy] acetic acid (etacrynic acid, EA), 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), 5-*N*,*N*-hexamethylene amiloride (NHA), D-glucuronic acid sodium salt, D-glutamic acid sodium salt, and nigericin were obtained from Sigma (St. Louis, MO). 2′,7′-bis-(2-carboxyethyl)-5,6-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was purchased from Molecular Probes Europe BV (Leiden, The Netherlands). $Na^{36}Cl^-$ (sp. act., 14–15 μCi/g Cl^-) was purchased from Amersham International (Amersham, U.K.).

Antibodies

The following murine mAbs have been used: mAb TS1-18 (IgG1), recognizing the CD18 subunit (common β-chain) of the CD11/CD18 Ag complex (β_2 integrins) (23); mAb 24 (IgG1), directed against the Mg^{2+}-dependent, activation-specific epitope 24 present on all three leukocyte α subunits (20, 24); and mAb CBRM1/5 (IgG1), recognizing the activation-specific epitope CBRM1/5, localized to the I domain on the α-chain of CD11b/CD18 (Mac-1) (21). mAbs TS1-18 was affinity purified from ascite fluids recovered from mice injected with the corresponding cell lines obtained from the American Type Culture Collection (Manassas, VA); mAb 24 and mAb CBRM1/5 were donated by Dr. N. Hogg (Imperial Cancer Research Fund, London, U.K.) and Dr. T. A. Springer (Harvard Medical School, Boston, MA), respectively. A FITC-labeled rabbit anti-mouse IgG $F(ab')_2$ from Sigma (St. Louis, MO) was used as secondary Ab in immunofluorescence flow cytometry studies.

PMN isolation

PMN were isolated according to the method described by Metcalf et al. (25), with slight modifications. Briefly, 4 ml of fresh peripheral blood, collected in EDTA, was layered onto a discontinuous gradient of 62 and 75% Percoll in PBS and centrifuged at 200 × *g* for 10 min and then at 400 × *g* for another 15 min. After centrifugation, neutrophils collected at the interface between the 62 and 75% Percoll were taken and washed once in HEPES buffer (145 mM NaCl, 5 mM KCl, 5 mM HEPES buffer, 5 mM glucose, and 0.2% BSA). After a 10-s lysis with a hypotonic solution to eliminate contaminating erythrocytes, isolated PMN were washed again in HEPES buffer and then resuspended in the same medium at the desired concentration. The resulting cell population contained 95–97% neutrophils, 2–3% eosinophils, and 1–2% mononuclear cells. Unless otherwise stated, $CaCl_2$ and $MgCl_2$ (final concentration of both cations, 1 mM) were added to the cell suspension just before the start of the functional assays. The effect of inhibitors used in this study was analyzed in PMN pretreated for 10 min with the different compounds.

Preparation of FN- and FBG-coated surfaces

The coating of surfaces with FN or FBG was performed as previously described (26). Briefly, 50-μl aliquots of either 20 μg/ml FN or 400 μg/ml FBG dissolved in PBS were deposited into replicate flat-bottom microtiter plate wells (MaxiSorp Immuno microwell plates, 442404; Nunc, Roskilde, Denmark). The plates were then left at 37°C for 1 h in a humidified incubator. Just before use, the wells were washed three times with PBS.

Assay of adherence

The adherence of PMN to FN- or FBG-coated surfaces was evaluated exactly as previously described (9). The quantification of adherent PMN was performed by an enzymatic assay based on the measurement of myeloperoxidase activity.

Immunofluorescence flow cytometry

Surface expression of epitope 24 was measured in PMN suspended at 2 × 10^6/ml in HEPES buffer containing 1 mM $MgCl_2$ and 5 μg/ml mAb 24. The mAb was added to the cell suspension during the last 10 min of incubation at 37°C without or with TNF. At the selected times, cells were diluted in PBS, washed twice, and then incubated for another 45 min with a FITC-labeled rabbit anti-mouse IgG $F(ab')_2$ at room temperature. Cells were washed again in PBS, and resuspended in 1% formaldehyde. To assay the expression of epitope CBRM1/5 and CD18, the cell suspensions were cooled on completion of the incubation at 37°C. PMN were then incubated for 1 h at 4°C with mAb CBRM1/5 (15 μg/ml) or mAb TS1–18 (5 μg/ml). The cells were then washed free of the mAbs in ice-cold PBS and subsequently incubated with the FITC-labeled secondary Ab. After two washings in PBS, the cells were suspended in 1% formaldehyde. Samples were analyzed by using either a XL2 (Coulter Instrumentation; Fondazione Callerio, Trieste, Italy) or a Epics Elite (Coulter Instrumentation; Servizio di Immunoematologia e Trasfusione del Sangue, settore Tipizzazione tessutale, Ospedale di Cattinara, Trieste, Italy) flow cytometer.

Measurement of $^{36}Cl^-$ efflux from PMN in suspension

PMN, suspended at 10–15 × 10^6/ml in Ca^{2+}- and Mg^{2+}-free HEPES buffer, were loaded with $^{36}Cl^-$ as previously described by Simchowitz and De Weer (16) by incubating the cells with $^{36}Cl^-$ (3.0 μCi/ml) for 2 h at 37°C in a shaking water bath to equilibrate the radiotracer between the intracellular and extracellular compartment. After loading, the cells were washed twice with prewarmed unlabeled buffer to remove the tracer and suspended in the same medium at 2.5 × 10^6/ml. Measurements of $^{36}Cl^-$ efflux was performed by incubating the cells without or with TNF. At the desired times, 800-μl aliquots of cell suspension were collected from duplicate tubes and centrifuged for 30 s at 12,000 × *g*. Then, 750-μl aliquots of the supernatants were withdrawn, and their radioactivity was counted by liquid scintillation counting in a β counter (LS6000TA; Beckman Instruments, Fullerton, CA). The percentage of efflux was calculated as follows: [(cpm in the supernatant of $time_x$ sample) − (cpm in the supernatant of t_0 sample)]/[(total cpm of cell suspension) − (cpm of t_0 supernatant)] × 100.

Measurement $^{36}Cl^-_i$ of PMN

PMN were suspended at 15–20 × 10^6/ml in HEPES buffer and incubated with 2.5–3 μCi/ml $^{36}Cl^-$ for 2 h at 37°C. After loading, the cells were used without washing them free of the tracer. At the selected times of incubation without or with TNF, 200 μl of the cell suspensions were collected from duplicate tubes, diluted into 1400 μl of HEPES buffer prewarmed at 37°C, and centrifuged for 15 s at 12,000 × *g*. The pellet was suspended in 60 μl of HEPES buffer, and 50-μl aliquots were diluted into 500 μl of the same buffer layered on 600 μl of a mixture (ratio 4:1, density 1.005 ± 0.001 g/l) of silicone oil (density 1.041 g/L; Silicone AR 200 fluid, 200 centistobes; Serva Electrophoresis GmbH, Heidelberg, Germany) and paraffin oil (density: 0.873 g/L; Merck). After 1-min centrifugation at 12,000 × *g*, the supernatants were discarded, the bottom of the tubes were cut, and the cell pellet-associated radioactivity was counted as described above. The $^{36}Cl^-$ that remained associated to PMN at the selected incubation time was expressed as a percentage of $^{36}Cl^-$ associated to PMN at $t = 0$.

Measurement of pH_i

PMN pH_i was assayed by fluorescence spectrophotometry using cells loaded with the pH-sensitive dye BCECF-AM as previously described by Grinstein et al. (27). Briefly, cells were incubated at 8–10 × 10^6/ml with

5 μM BCECF-AM for 30 min at 37°C in a shaking water bath; they were washed twice with prewarmed PBS to remove the dye, and resuspended at 2.5–5 $\times$ 10^6 cells/ml in HEPES buffer. Then, 1 ml of cell suspension was transferred to a cuvette thermostated at 37°C under continuous stirring and analyzed using a 650-10S fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT). The nigericin/K^+ method described by Thomas et al. (28) was used to calibrate pH_i.

Results

Conformational changes are involved in TNF-induced functional activation of PMN β_2 integrins

Table I shows that PMN at rest poorly adhere to immobilized FN or FBG, and that TNF strongly enhances the adhesive response on both biologic surfaces. TNF-stimulated adhesion mostly relies on β_2 integrin engagement because it is inhibited by the anti-CD18 mAb TS1-18 and omission of Mg^{2+}, a cation that is required for the full activation of β_2 integrins (29, 30). Pretreatment of PMN with 250 μM DIDS, a drug that blocks increased CD18 surface expression in PMN exposed to several agonists, including TNF (8, 31), does not modify the adherence to FN of TNF-stimulated cells (60.4 $\pm$ 9.5% adherence of DIDS-pretreated cells vs 67.2 $\pm$ 12.5% adherence of untreated cells, mean $\pm$ SEM, $n = 7$; $p = 0.3$, Student's t test on paired data), supporting the notion that acquirement of an "active" state, rather than an increase in quantitative expression, is crucial for an optimal β_2 integrin functional activity (31–37).

To address the question as to whether TNF-induced activation of β_2 integrins is accounted for by conformational changes of the heterodimers, we measured the surface expression of epitope 24, an activation-specific epitope present on each of the three α subunits of functionally active β_2 integrins (20). Table II shows that TNF effectively promotes the transition of β_2 integrins from an "inactive" to an "active" conformation, because the surface expression of epitope 24 was much higher in TNF-treated cells than in untreated cells. Such an increase in epitope 24 expression cannot be ascribed to an aspecific binding of mAb 24, because this mAb does not bind to PMN incubated with the cytokine under conditions known to prevent the expression of epitope 24, such as 0°C or in Mg^{2+}-free buffer (20). The finding that TNF enhances the expression on PMN of the CBRM1/5 epitope, another integrin "activation reporter" localized to the I domain on the α-chain of Mac-1 (21) (15.7 $\pm$ 4.0 of TNF-treated PMN vs 5.1 $\pm$ 2.6 of untreated PMN; mean $\pm$ SEM of mean channel fluorescence values, $n = 4$) further demonstrates the ability of the cytokine to promote conformational rearrangements of β_2 integrin structure.

Table I. *TNF-stimulated PMN adherence to FN and FBG depends on β_2 integrin engagement*[a]

PMN Treatment	% Adherence	
	FN	FBG
None	6.8 $\pm$ 1.6	6.0 $\pm$ 2.6
TNF	63.6 $\pm$ 4.2	68.5 $\pm$ 5.2
TNF + mAb TS1-18	18.5 $\pm$ 3.5	21.2 $\pm$ 3.0
TNF in Mg^{2+}-free buffer[b]	12.0 $\pm$ 1.9	14.1 $\pm$ 2.6

[a] PMN (1.5 $\times$ 10^6/ml in HEPES buffer) were prewarmed in suspension at 37°C for 5 min. Afterward, TNF (10 ng/ml final concentration) and mAb TS1-18 (10 μg/ml final concentration) were added, where indicated, to the incubation mixtures. After a further 10-min incubation, $CaCl_2$ and $MgCl_2$ (final concentration of both cations 1 mM) were added, and 50 μl of the cell suspensions were then transferred to FN- or FBG-coated wells containing 1 mM of $CaCl_2$ and $MgCl_2$ in 100 μl of prewarmed HEPES buffer. After 20 min of incubation at 37°C in a humidified incubator, nonadherent PMN were removed, and quantification of adherent cells was performed as previously described by means of an enzymatic assay based on the measurement of myeloperoxidase activity (9). Data represent the means $\pm$ SD of three to five experiments performed in duplicate with PMN isolated from different donors.

[b] PMN were maintained in HEPES buffer containing 1 mM $CaCl_2$ and 1 mM EDTA.

Cl^- transport blockers inhibit the TNF-induced β_2 integrin activation

The expression of activation-specific neoepitopes on the leukocyte surface has been suggested to reflect conformational changes that propagate from the integrin cytoplasmic domain to the extracellular domains (20, 21, 36, 37). Starting from our previous observation that a massive net Cl^- efflux regulates TNF-stimulated PMN adherence to FN (9), we asked the question whether a drop in Cl^-_i might serve as a potential mechanism whereby TNF induces the conformational changes underlying β_2 integrin activation. To this end, we tested the effect of two drugs belonging to two unrelated families of Cl^- transport blockers, namely, EA and 2-aminomethyl-4-(1-methyl-1-phenylethyl)-6-iodophenol hydrochloride (MK447/A; Refs. 16, 18, 38), on both Cl^- efflux and epitope 24 expression in TNF-stimulated PMN. PMN pretreated with either inhibitor release less Cl^- (Fig. 1*a*) and, interestingly, exhibit a lower expression of epitope 24 compared with untreated cells (Fig. 1, *b–d*). In line with these results, TNF-stimulated adherence of EA- or MK447/A-treated PMN to FN and FBG was found to be markedly inhibited (Fig. 1*e*). These findings speak in favor of the hypothesis that the TNF-induced activation of β_2 integrins occurs through conformational changes that are under the control of PMN Cl^-_i.

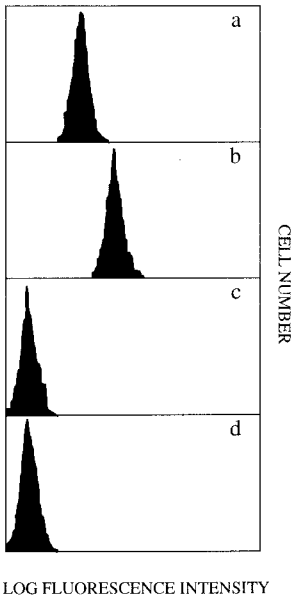
Unstimulated PMN suspended in Cl^--free buffers release Cl^-, show an increased expression of epitope 24, and adhere to FBG-coated surfaces

Further evidence of the involvement of Cl^-_i in the regulation of β_2 integrin activation was obtained by using an alternative experimental approach, which explored the possibility that Cl^- efflux triggered by means other than TNF treatment would mimic the drop in Cl^-_i and β_2 integrin conformational changes induced by the cytokine. To this end, Cl^- efflux and epitope 24 expression were measured in unstimulated PMN suspended in a Cl^--free buffer, i.e., a buffer where Cl^- was replaced by the cell-impermeant anion glutamate. As expected, PMN suspended in such buffer, but not those suspended in the usual Cl^--containing buffer, exhibit a massive Cl^- efflux (55.9 $\pm$ 5.0% of $^{36}Cl^-$ efflux of PMN in glutamate-containing buffer vs 17.2 $\pm$ 3.5% of $^{36}Cl^-$ efflux of PMN in Cl^--containing buffer, mean $\pm$ SEM, $n = 5$). This is accompanied by a marked up-regulation of epitope 24 expression (Fig. 2*a*) and an increased adhesion to FBG (Fig. 2*c*). Similar results were obtained with PMN suspended in a buffer containing another cell-impermeant anion, i.e., glucuronate, which has been previously shown to cause a decrease of PMN Cl^-_i (9, 39) (Fig. 2, *b* and *c*). In the absence of Mg^{2+}, PMN suspended in Cl^--free buffers do not show any appreciable up-regulation of epitope 24 expression (results not shown), thus ruling out a possible nonspecific effect of the buffers.

In the light of the above reported results, we tested adhesion and Cl^- efflux in PMN isolated from a subject who routinely showed an unusually high basal adherence of PMN to FBG. Interestingly, we found that the high basal cell adhesion was accompanied by a high basal Cl^- efflux (40.9% adherence and 68.5% Cl^- efflux), thus resembling the response of control PMN placed in Cl^--free buffer (45.6% adherence and 59.8% Cl^- efflux). In the same experiment, adherence and Cl^- efflux of control PMN in Cl^--containing buffer were 5.3 and 12.9%, respectively. This finding adds evidences to the existence of a positive relationship between a drop in Cl^-_i and the activation of β_2 integrins.

Table II. *Expression of epitope 24 on PMN*[a]

PMN treatment	Mean channel fluorescence
None	22.1 ± 8.3
TNF	85.9 ± 9.9[b]
TNF, 0°C[c]	0.7 ± 0.33
TNF in Mg^{2+}-free buffer[d]	0.33 ± 0.1



[a] PMN (2×10^6/ml in HEPES buffer containing 1 mM Mg^{2+}) were prewarmed in suspension at 37°C for 5 min in a shaking water bath. After addition of TNF (final concentration 10 ng/ml) or an equal volume of buffer, the cells were incubated for another 30 min. mAb 24 (final concentration 5 μg/ml) was added to the incubation mixture during the last 10 min of incubation. All the subsequent procedures, including washings in PBS and incubation with a FITC-labeled rabbit anti-mouse IgG $F(ab')_2$, were performed at room temperature. The cells were finally suspended in 1% formaldehyde and analyzed by flow cytometry. Data show values of mean channel fluorescence after subtraction of background fluorescence (12.2 ± 3.3) and represent the means ± SEM of three to seven experiments. Panels *a–d* show histograms from one representative experiment.

[b] $p < 0.0001$ vs untreated cells, Student's *t* test on paired data, $n = 7$.

[c] After addition of TNF, the cells were maintained at the ice-melting temperature throughout the whole experimental procedure.

[d] PMN were suspended in Mg^{2+}-free HEPES buffer containing 1 mM EDTA.

Restoration of the basal Cl^-_i levels reverses β_2 integrin activation

The TNF-induced drop in PMN Cl^-_i is a reversible phenomenon because basal Cl^-_i levels can be restored upon prolonged incubation (45–60 min) (18). Thus, if our hypothesis of a positive relationship between decrease in Cl^-_i and increase in β_2 integrin activation is correct, it would be reasonable to expect that, upon Cl^- regaining, the conformational changes underlying the active state of the heterodimers should be reversed. This is, indeed, the case, as shown in Fig. 3*a*. The maximal drop in Cl^-_i (after a 30-min incubation with TNF) is accompanied by a marked increase in the expression of epitope 24 (see also Fig. 3*c*), whereas the subsequent 30-min incubation restores Cl^-_i and decreases epitope 24 expression (see also Fig. 3*d*). This decrease in surface expression of epitope 24 is not accounted for by a decrease in the expression of the β_2 integrin heterodimers, because CD18 expression augments up to 20–30 min of incubation with TNF and does not change by prolonging the incubation to 60 min (Fig. 3, *e–g*). In line with these results are the data of experiments in which the adhesive response of PMN was assayed. The percentage of cells that adhere to FN after a 30-min incubation in suspension with TNF (33.18 ± 6.5; mean ± SD, $n = 3$) decreases at 60-min incubation (13.1 ± 3.1; mean ± SD, $n = 3$), suggesting that a down-regulation of β_2 integrin activation has occurred. It must be pointed out that the decreased response of PMN exposed to TNF for 60 min cannot be ascribed to a cytotoxic effect of the cytokine, because 1) the percentage of apoptotic cells in TNF-treated PMN suspension is almost the same as in resting PMN (7.9 ± 3.5% of TNF-treated PMN vs 8.5 ± 1.9% of resting PMN, mean ± SEM of three to five examinations of randomly chosen microscopic fields) and 2) upon subsequent exposure to 10^{-7} M FMLP, TNF-treated cells show a marked increase in adhesion to FN-coated surfaces, which is strictly comparable to that of untreated cells (data not shown).

An additional experimental approach was used to strengthen the hypothesis that changes in Cl^-_i may regulate β_2 integrin activation. Fig. 4*a* shows that PMN, exposed for 30 min to TNF in Cl^--containing buffer, washed, and then suspended in Cl^--free buffer for another 30 min, exhibit a further drop in Cl^-_i accompanied by a marked increase in epitope 24 expression (Fig. 4*b*). A similar increase also occurs if the same treatment, i.e., washing and suspension in Cl^--free buffer, is applied to PMN exposed to TNF for 60 min (Fig. 4, *a* and *b*). It is worthy of note that up- and down-regulation of epitope 24 expression can be easily reproduced in unstimulated PMN when fluctuations of their Cl^-_i are induced by solely altering the Cl^- content of the buffer (Fig. 4, *c* and *d*).

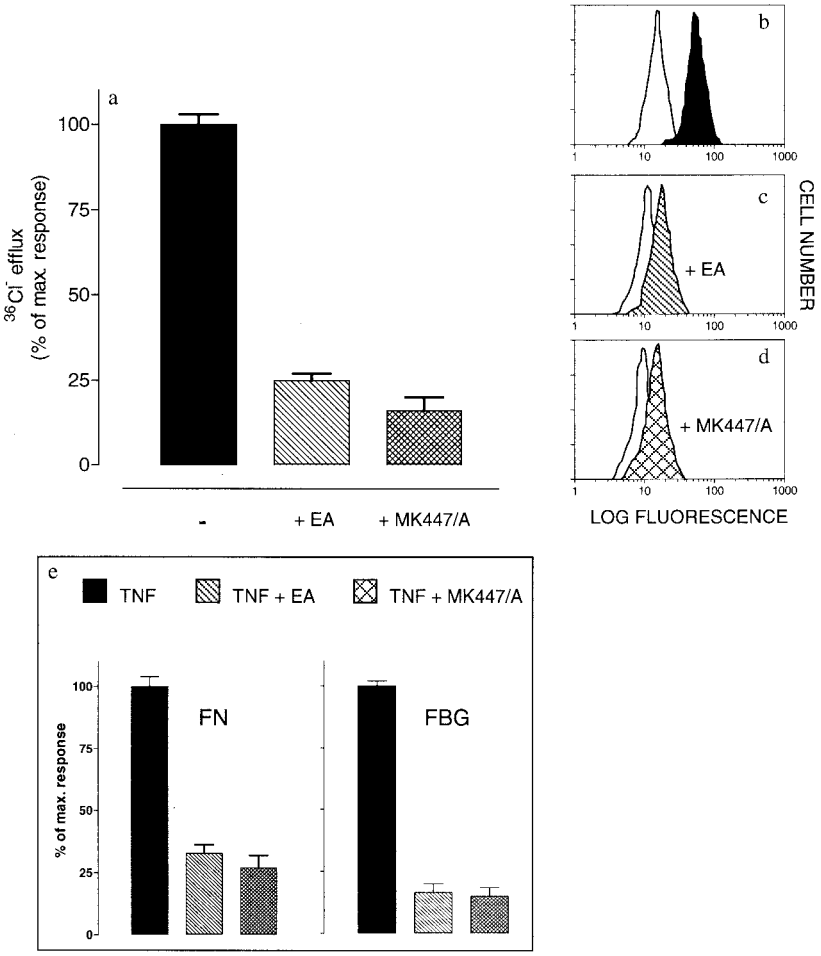


FIGURE 1. Cl^- transport blockers inhibit Cl^- efflux, adherence, and epitope 24 expression in TNF-stimulated PMN. Aliquots of $^{36}Cl^-$-loaded PMN (2×10^6/ml in HEPES buffer) were preincubated in suspension for 10 min at 37°C without or with EA or MK447/A (final concentration, 250 and 150 μM, respectively). At the end of the preincubation, $CaCl_2$ and $MgCl_2$ (final concentration of both cations, 1 mM) were added to duplicate aliquots of the cell suspensions to be used in the assays of Cl^- efflux (*a*) and adherence to FN- or FBG-coated surfaces (*e*). $MgCl_2$ only was added to cell suspensions used in the assay of epitope 24 expression (*b–d*). After addition of TNF (final concentration 10 ng/ml), PMN were incubated for 20 min at 37°C in a shaking water bath. To assay $^{36}Cl^-$ efflux, aliquots of the cell suspension were collected and centrifuged for 30 s at 12,000 $\times$ *g*. The supernatants were then counted for radioactivity as described in *Materials and Methods*. To assay PMN adherence, aliquots of the cell suspensions were collected and transferred to FN- or FBG-coated wells of a microtiter plate. The plate was then incubated in a humidified incubator for 15 min at 37°C. On completion of the incubation, nonadherent PMN were removed by centrifuging the plate upside-down, and the number of adherent cells was determined by assaying myeloperoxidase activity (9). Data of $^{36}Cl^-$ efflux and adherence are the means ± SEM of seven experiments. Assay of epitope 24 expression was as described in Table II. mAb 24 (final concentration, 5 μg/ml) was added to the cells' suspensions during the last 10 min of incubation without (white histograms in *b–d*) or with TNF. Histograms in *b–d* show one experiment representative of four that gave similar results.

Taken together, these results suggest that Cl^-_i changes modulate β_2 integrin activation and indicate that the conformational rearrangements underlying TNF-induced activation of β_2 integrins is transient.

Mutual relationships between changes in Cl^-_i and pH_i induced by TNF

It has been shown that, in TNF-stimulated PMN, the onset of Cl^- efflux correlates with the onset of a cytoplasmic alkalinization (18). We thus addressed the issue as to whether the increase in cytoplasmic pH is in some way related to TNF-induced Cl^-_i decrease and, if so, whether such cytoplasmic alkalinization is involved in the process of β_2 integrin activation. Fig. 5 shows that TNF-induced Cl^- efflux (*upper panel*) is accompanied by a concomitant rise in pH_i (*lower panel*). Such a rise is likely mediated by the activation of the Na^+/H^+ exchanger, because the response is abolished by the amiloride analog NHA, a potent and specific inhibitor of the exchanger (40) (*lower panel*). NHA does not affect Cl^- efflux (*upper panel*), but the Cl^- transport blockers MK447/A and EA markedly impair the TNF-induced alkalinization (*lower panel*), suggesting that the activation of the Na^+/H^+ exchanger may be under the control of the cytoplasmic levels of Cl^-. The inhibitory effect of the two Cl^- transport inhibitors on the TNF-induced alkalinization (Fig. 5, *lower panel*) is not accounted for by toxic effect of the drugs toward the Na^+/H^+ exchanger because, upon addition of a saturating dose of PMA (20 ng/ml), MK447/A- and EA-treated PMN show a normal alkalinizing response (data not shown). In agreement with these findings are the results of parallel experiments showing that NHA does not alter the TNF-

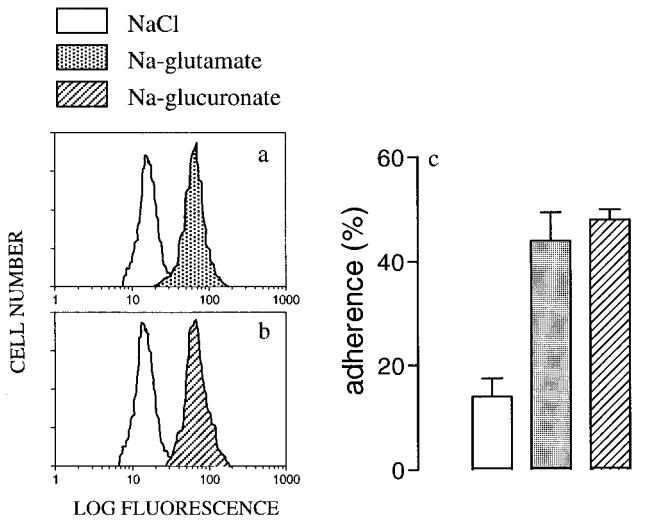


FIGURE 2. Effect of Cl^--free buffers on epitope 24 expression and adherence to immobilized FBG of unstimulated PMN. After loading with $^{36}Cl^-$, aliquots of PMN were extensively washed in prewarmed unlabeled HEPES buffer. The pellets were then suspended in either glutamate- or glucuronate-containing buffer. Assay of epitope 24 expression was as described in Table II. Adherence to FBG-coated surfaces was assayed as described in Fig. 1. *a* and *b*, Histograms from one experiment of epitope 24 expression representative of three. Data of adherence (*c*) represent the means ± SEM of three to five experiments (in duplicate) performed with cells isolated from different donors.

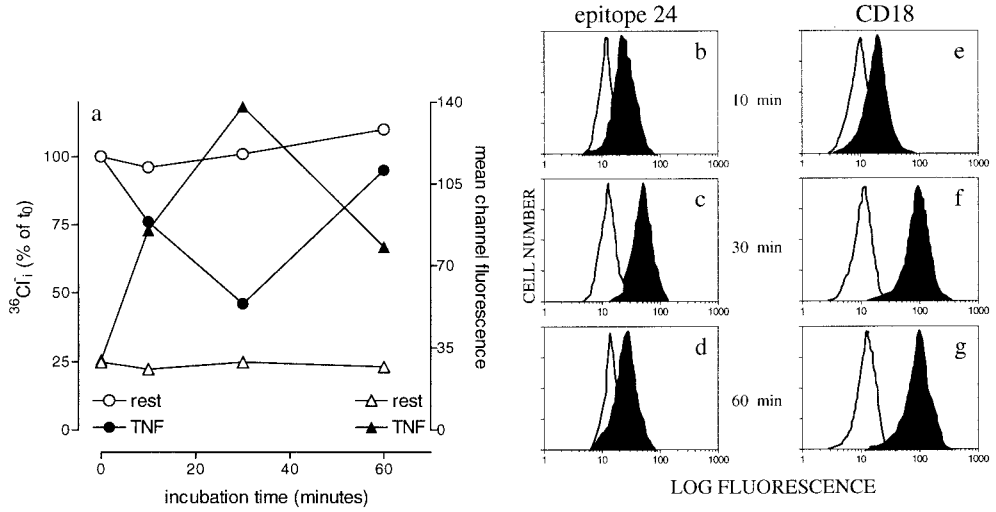


FIGURE 3. Relationship between changes in $^{36}Cl^-_i$ and surface expression of epitope 24 and CD18 in resting and TNF-stimulated PMN. To assay $^{36}Cl^-_i$, which reflects net Cl^- movements, TNF (final concentration, 10 ng/ml), or the appropriate control solution, was added to $^{36}Cl^-$-loaded PMN maintained in the hot loading medium. At selected times, aliquots of the cell suspension were collected from duplicate tubes, diluted into prewarmed unlabeled HEPES buffer, and centrifuged for 15 s at 12,000 × *g*. The pellet was suspended in 60 μl of HEPES buffer, and 50-μl aliquots were added to 450 μl of the same buffer layered on 600 μl of a mixture of silicone oil and paraffin oil (ratio, 4:1; density, 1.005 ± 0.001 g/L). After 1-min centrifugation at 12,000 × *g*, the supernatants were discarded, the bottom of the tubes were cut, and the cell pellet-associated radioactivity was counted. Assay of epitope 24 expression was as described in Table II. CD18 expression was measured by immunofluorescence flow cytometry as described in detail in *Materials and Methods*. Each point in the graphs shown in (*a*) represents the mean of two experiments (in duplicate) performed with cells isolated from different donors. Data of $^{36}Cl^-_i$ (○, resting; ●, TNF) and epitope 24 expression (values of mean channel fluorescence intensity: △, resting; ▲, TNF) refer to left and right ordinate, respectively. *b–d* and *e–g*, Histograms of one representative experiment of epitope 24 and CD18 expression, respectively. White histograms show the expression of epitope 24 and CD18 in unstimulated PMN.

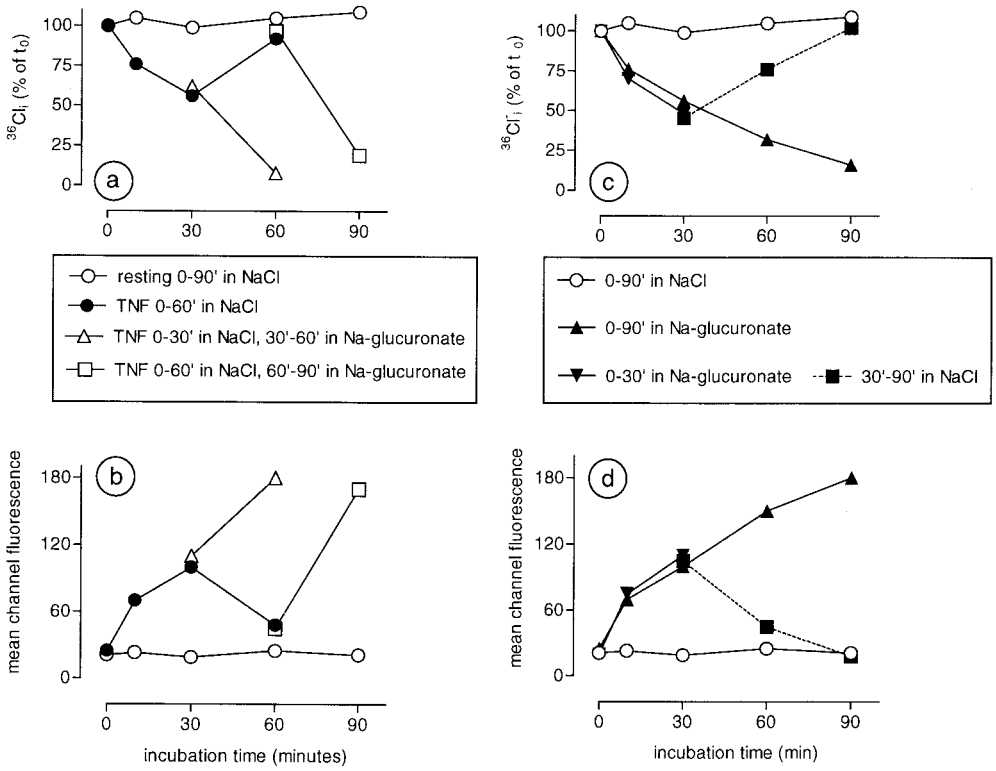


FIGURE 4. Effect of Cl^--free buffer on changes in $^{36}Cl^-_i$ and epitope 24 expression of TNF-stimulated or unstimulated PMN. *a* and *b*, After 30 and 60 min of incubation with TNF (final concentration, 10 ng/ml) in NaCl-containing HEPES buffer, aliquots of the cell suspensions were centrifuged and suspended in Na-glucuronate-containing buffer. *c* and *d*, Unstimulated PMN were maintained in either NaCl- or Na-glucuronate-containing HEPES buffer for 90 min. After a 30-min incubation, aliquots of the cell suspended in glucuronate-containing buffer were centrifuged and resuspended in NaCl-containing buffer. At selected times, the cells were assayed for $^{36}Cl^-_i$ (*a* and *c*) and epitope 24 expression (*b* and *d*) as described in Fig. 3 and Table II, respectively. Each point in the graphs represents the mean of two experiments (in duplicate) performed with cells isolated from different donors.

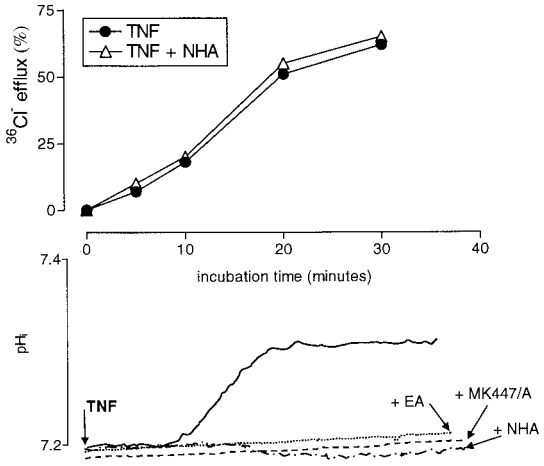


FIGURE 5. Relationships between Cl^- efflux and pH_i changes of TNF-stimulated PMN. To assay $^{36}Cl^-$ efflux (*upper panel*), aliquots of $^{36}Cl^-$-loaded PMN (2×10^6/ml in HEPES buffer) were preincubated in suspension in polypropylene test tubes for 10 min at 37°C without or with NHA (final concentration, 1 μM). At the end of the preincubation, TNF (final concentration, 10 ng/ml) was added to the cell suspensions. At selected times, aliquots of the incubation mixtures were withdrawn and assayed for Cl^- efflux as described in the legend to Fig. 1. To assay pH_i changes (*lower panel*), BCECF-AM (final concentration, 5 μM) was added to aliquots of PMN suspension during the last 30 min of the incubation used to load the cells with $^{36}Cl^-$. After two washing steps in prewarmed unlabeled HEPES buffer, the cells were counted and suspended at 5×10^6/ml in the same buffer. Then, an 800-μl aliquot of the cell suspension was withdrawn in a thermostated cuvette placed in a spectrophotofluorometer equipped with a device for continuous stirring of the incubation mixture. Changes in pH_i were monitored upon addition of TNF (final concentration, 10 ng/ml) (*arrow*). To assay the effect of NHA, EA, and MK447/A (final concentrations, 1, 250, and 150 μM, respectively), the cells were preincubated with the required compound for 10 min at 37°C in the cuvette under continuous stirring. The nigericin/K^+ method described by Thomas et al. (28) was used to calibrate pH_i. Each point in the graphs shown in the *upper panel* represents the means of duplicate assays of one experiment representative of three that gave similar results. Traces shown in the *lower panel* are representative of three similar experiments. Abscissa axis refers to both upper and lower panel.

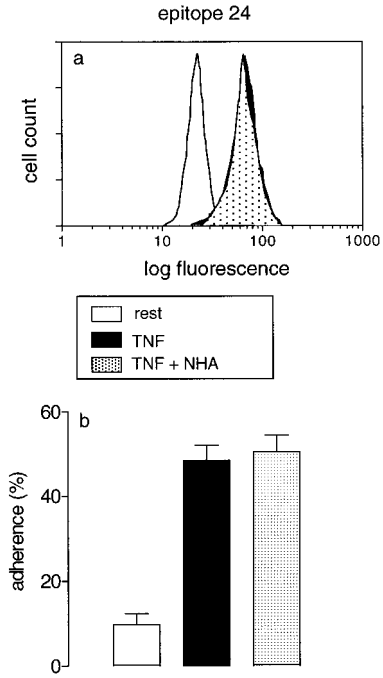


FIGURE 6. TNF-induced β_2 integrin activation does not depend on cytoplasmic alkalinization. Aliquots of $^{36}Cl^-$-loaded PMN were preincubated in suspension for 10 min at 37°C with NHA (final concentration 1 μM). After addition of TNF (final concentration, 10 ng/ml), the suspensions were incubated for 20 min at 37°C. Assays of epitope 24 expression (*a*) and adherence to immobilized FBG (*b*) were as described in Table II and Fig. 1, respectively. Histograms in (*a*) are representative of three similar experiments. Data of PMN adherence are the means ± SEM of three to five experiments (in duplicate).

induced expression of epitope 24 (Fig. 6*a*). Moreover, PMN pretreated with NHA and then exposed to TNF, adhere to FBG to the same extent of untreated cells (Fig. 6*b*). These findings strongly suggest that a rise in pH_i is not required for the activation of β_2 integrins.

Discussion

The process of β_2 integrin activation, which enhances the ability of these heterodimers to interact with ligands (32, 33, 36, 37, 41, 42), plays a pivotal role in TNF-induced, adherence-dependent PMN responses (1–10). This study was stimulated by the following apparently unrelated observations 1) activation, rather than increased expression, is crucial for an optimal β_2 integrin functional activity (31–37); 2) PMN respond to TNF with an early and marked drop in their unusually high basal Cl^-_i (18); and 3) changes in Cl^-_i have been shown to be involved in the regulation of some PMN functional responses (9, 19, 43, 44). Our results somehow lay a bridge between the fields of integrin research and ion research showing that TNF regulates the functional activity of neutrophil β_2 integrins by reversibly modulating their "active" state through conformational changes that depend on alterations of the cellular Cl^- content.

It has been suggested that the propagation of conformational changes from the integrin cytoplasmic domain to the extracellular domains (20, 21, 36, 37) leads to the appearance of "activation-specific neoepitopes", the recognition of which, with specific mAbs, is exploited to assay integrin activation (20, 21, 45, 46). The results showing that TNF increases the expression of epitopes 24 and CBRM1/5, two distinct "activation reporters" of β_2 integrins (20, 21), allow to include the cytokine in the as yet small group of physiological signaling molecules that directly induce conformational changes of β_2 integrins together with ADP (47, 48), L-selectin (49), and IL-8 (21).

How TNF promotes the conformational rearrangements that account for the increased expression of the aforementioned epitopes is of great interest. In fact, identification of the mechanisms by which the cytokine activates the integrins is an essential prerequisite for a possible pharmacologic control of TNF-induced adherence-dependent PMN responses (1–9, 13, 50). A new contribution to this issue comes from the results reported in the second part of this study, which show that TNF-induced β_2 integrin activation is controlled by alterations of the cellular content of chloride. This is suggested by that fact that 1) TNF-induced β_2 integrin conformational changes are accompanied by a drop in Cl^-_i, and 2) inhibition of such a drop prevents β_2 integrin activation.

The existence of a positive relationship between TNF-induced alterations in Cl^-_i and changes in β_2 integrin functional activity is further strengthened by the finding that Cl^- movements are involved not only in the up-regulation, but also in the down-regulation of β_2 integrin activation. In fact, the restoration of the PMN basal Cl^-_i levels, occurring via an as yet poorly defined "Cl^- regaining mechanism" (9, 18), is accompanied by a decrease in

both the levels of epitope 24 expression (Fig. 3) and cell adhesive response. In the light of these results, one would expect the conditions that prevent Cl^- regaining to also prevent β_2 deactivation. Indeed, this is what happened in the model, reported previously (9), with PMN stimulated by TNF while residing on FN-coated surfaces, where a positive relationship was shown to exist between the decrease in Cl^-_i and some PMN functional responses (e.g., adherence, cytoskeleton reorganization, and NADPH-oxidase assembly). In such a model, the Cl^- regaining mechanism does not work and β_2 integrin activation does not undergo down-regulation, as suggested by the finding that the percentage of adherent PMN reached a maximum after 40–60 min of incubation (9) and remained unchanged up to 120 min (our unpublished observation).

The use of Cl^--free buffers, which induce PMN Cl^- efflux down its concentration gradient, has allowed us to further highlight the role of Cl^-_i changes in the regulation of β_2 integrin activation. In fact, this paper demonstrates that changes in β_2 integrin conformation can be easily reproduced via manipulation of the Cl^- content of the buffer where PMN are suspended (Figs. 2 and 4).

A further issue addressed by this study concerns the interrelationships between changes in Cl^-_i and pH_i in TNF-stimulated PMN. Indeed, the previously reported observation that Cl^- efflux and cytoplasmic alkalinization are concomitant responses of PMN to TNF (18) allowed for the possibility that a rise in pH_i might also be involved in the regulation of β_2 integrin activation. Cross-inhibition experiments (Figs. 5 and 6), on the one hand, indicate that a cytoplasmic alkalinization is not involved in the expression of activation-specific neoepitopes of β_2 integrins and, on the other hand, suggest a role for the TNF-induced drop in Cl^-_i in the activation of the Na^+/H^+ exchanger and thus in the control of cytoplasmic pH (51, 52). It is worth remembering that, as opposed to the TNF-induced Cl^- efflux, the Cl^- efflux triggered by β_2 integrin cross-linking is dependent on a rise in pH_i (53), providing additional proof of the complexity of the mechanisms that control ion fluxes in PMN. Therefore, it is conceivable that at least two different Cl^- outward transport mechanisms may operate in PMN, a pH_i-independent one, triggered by TNF and possibly other soluble agonists, and a pH_i-dependent one, switched on by β_2 integrin cross-linking. The observation that TNF- and β_2 integrin-induced Cl^- efflux display different sensitivity to Cl^- transport blockers (R. Menegazzi, manuscript in preparation) is compatible with this hypothesis.

Although the role of integrins as bidirectional transducers of transmembrane signals (i.e., both "inside-out" and "outside-in" signals) is well established (reviewed in Ref. 54), the molecular mechanisms that control such integrin-dependent signaling are still under active investigation. In a previous paper, we showed that the Cl^- efflux triggered by β_2 integrin cross-linking has a role in the outside-in transmembrane signaling that regulates adherence-dependent PMN responses, such as spreading and respiratory burst (53). Interestingly, the results of this study provide evidence that the changes in Cl^-_i may be included also among the mechanisms that control an inside-out signal, i.e., the modulation of β_2 integrin activation.

How Cl^- movements can promote the conformational changes that feature the "active" or the "inactive" state of the integrins (29) remains to be elucidated. It has been shown that an electrostatic interaction between acidic and basic amino acids, located in the membrane-proximal regions of the α and β subunit cytoplasmic domains of the $\alpha_{IIb}\beta_3$ integrin, constrains this heterodimer in an inactive conformation (55). Moreover, point mutations experiments show that the disruption of this potential salt bridge leads to dissociation of the two subunits with consequent activation of the integrin. A similar phenomenon has been shown to regulate LFA-1 (CD11a/CD18) activation in K562-transfected cells (56). Thus, the hypothesis should be considered that alterations of the cytoplasmic levels of Cl^- can somehow influence the possible interactions between the membrane-proximal regions of the α and β subunit cytoplasmic domains of β_2 integrins. According to this hypothesis, the regulation of the ligand binding activity of β_2 integrins might simply rely on the combined action of inorganic ions: Cl^-, which acts from within the cell, and Mg^{2+}, which binds to the extracellular domains of the α subunits (20, 57, 58). Interestingly, other authors provided evidence for the involvement of Cl^- in the modulation of protein function, such as angiotensin-converting enzyme (59), yeast aminopeptidase I (60), hormone-sensitive GTP-dependent regulatory proteins (61), and, more recently, cathepsin C (62).

The meaning of the unusually high basal Cl^-_i of PMN is as yet unclear, but a growing number of papers in literature show that a drop in Cl^-_i is involved in many responses of these cells (9, 18, 19, 43, 44, 53). We believe that this study, demonstrating that alterations of Cl^-_i modulate β_2 integrin activation, contributes to the establishment of the role of Cl^- as a new PMN "second messenger".

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