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Expression of urokinase plasminogen activator receptor in resting and activated bovine neutrophils

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Summary. Changes in urokinase-plasminogen activator (u-PA) and u-PA receptor (u-PAR) expression at the protein and mRNA level in resting neutrophils and in neutrophils activated by phorbol myristate acetate (PMA) were examined. Low amounts of u-PA were found intracellularly or membrane-bound in resting neutrophils. However, incubation of resting neutrophils with purified exogenous u-PA (10 IU/ml) revealed extensive binding of u-PA to cell membranes. Excess amino-terminal fragment of the u-PA molecule, a proteolytically inactive fragment of u-PA (amino acids 1–135) blocked binding of exogenous u-PA to the cell membrane. These results, collectively, indicate that the binding of u-PA is specific and that resting neutrophils have unoccupied u-PA receptors on their cell membrane. Addition of PMA led to an increase ($P < 0{\cdot}01$) in total cell-associated, membrane-bound u-PA activity and u-PA mRNA expression by bovine neutrophils. In contrast, PMA increased u-PAR mRNA levels but this was accompanied by a decrease (2·5-fold; $P < 0{\cdot}01$) in free, unoccupied u-PA binding sites. No significant effects on total cell-associated or membrane-bound u-PA were found when neutrophils were treated with 4-phorbol 12,13 didecanoate, a phorbol ester that does not activate protein kinase C (PKC). Furthermore, addition of 1-(5-isoquinolinesylphonyl)-2-methylpiperazine dihydrochloride (H-7), a potent PKC inhibitor, blocked the effect of PMA on total cell-associated u-PA activity. Thus, PKC plays a role in the modulation of u-PA and u-PAR by PMA in bovine neutrophils.

Keywords: Urokinase receptor, bovine neutrophils.

Migration of neutrophils to an infected or injured tissue is the typical manifestation of an inflammatory response. Neutrophils start their migration by penetrating through blood capillaries, a process known as diapedesis. In the first stage of diapedesis, neutrophils, presumably guided by chemoattractants, come in contact with endothelial cells. Interaction of neutrophils and endothelial cells is facilitated by expression of numerous cell adhesion molecules expressed on the surface of both cell types (Klein & Horejsi, 1997).

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To breach the mechanical barrier imposed by the basement membrane, neutrophils may use limited proteolytic activity at regions of cell contact (Plesner *et al.* 1994). Neutrophils express a specific urokinase-plasminogen activator (u-PA) receptor (u-PAR, CD 87). Typically, u-PAR clusters are observed at the leading edges of migrating cells (Sitrin *et al.* 2000). u-PA can bind to this receptor and, thus, can convert the abundant proteolytically inactive proenzyme, plasminogen, to active plasmin. Plasmin, in turn, is capable of degrading certain matrix components, in addition to activating other matrix-degrading enzymes such as metalloproteinases (Politis, 2000). The presence and binding kinetics of u-PAR have been verified in several cell types but the presence and pattern of u-PAR expression has not been examined in detail in resting and activated bovine neutrophils.

Bovine u-PAR is a protein consisting of 310 amino acids and is highly glycosylated. u-PAR is attached to the plasma membrane by an anchor of glycosyl, phosphatidyl, and inositol (GPI anchor). u-PAR binds u-PA at the epidermal growth factor (EGF)-like domain, which is part of the amino terminal region of the u-PA molecule. The u-PA that is bound to the receptor can convert plasminogen to plasmin. Such plasmin formed on the cell membrane has properties different from those of plasmin present in solution. For example, plasmin on the membrane is slowly inactivated by plasmin inhibitors while complete inactivation of plasmin cannot be achieved (Politis, 1996).

Recent reports have added complexity to the u-PAR system because they indicate that u-PAR occupancy regulates cell migration in the absence of functional u-PA, in experiments performed using u-PA deficient mice (Waltz *et al.* 2000). This observation suggests that u-PAR has a protease-independent function *in vivo*. In further support of this suggestion, Gyetko *et al.* (2000) showed that u-PAR is important for recruitment of neutrophils to the lung and this requirement is independent of u-PA. They suggested a novel role for u-PAR as a modulator of beta-2-integrin, particularly CD3 (CD11b/CD18, Mac-1).

Observations that u-PA binding sites are rapidly distributed to the leading edges of migrating cells and that u-PAR number and affinity can be modulated by phorbol esters and cytokines (Blasi, 1993) suggest that u-PAR function is dynamic in nature. The question of how u-PAR is regulated in resting and activated bovine neutrophils has not been addressed. Furthermore, dependence of u-PAR expression on protein kinase C (PKC) in PMA-activated bovine neutrophils has not been studied.

Neutrophils can be activated following *in vitro* incubation with several agents such as ConA, phorbol myristate acetate (PMA), formyl-phenyl-leucyl-phenylalanine and tumor necrosis factor α. PMA is the most commonly used agent for activation of neutrophils (Heiple & Ossowski, 1986; Dore *et al.* 1992; Plesner *et al.* 1994; Lund *et al.* 1995; Takei *et al.* 1996; Allard *et al.* 1999).

The objectives of the present work were to study the pattern of expression of u-PA and u-PAR at the protein and mRNA level in resting neutrophils and in those activated by PMA. Furthermore, the dependence of changes in u-PA and u-PAR expression on protein kinase C in PMA-activated neutrophils was examined.

Materials and methods

Animals, blood collection, neutrophil isolation

Ten cows were used to supply blood for neutrophil isolation. Blood samples were collected monthly for a period of 4 months from all cows. Neutrophils were isolated as described by Politis *et al.* (1996). Briefly, 30 ml of heparinized (10 IU heparin/ml)

venous blood was layered onto 20 ml of a sodium metrizoate (95 g/l)-polysaccharide gradient (56 g/l) with a specific gravity of 1·077 g/cm^3 (Sigma Chemical Co., St. Louis, MO 63178), then centrifuged at 500 ***g*** for 45 min. Cells from the bottom layer were washed twice in RPMI-1640 medium (Sigma) and red blood cells were lysed by the addition of 20 ml sterile distilled water, followed by the addition of sterile saline solution (27 g NaCl/l) to restore isotonicity. Cells were pelleted by centrifugation at 2000 ***g*** for 10 min and resuspended in RPMI-1640 medium containing fetal bovine serum (FBS; 100 ml/l; Sigma) at a concentration of 1×10^7 cells/ml. Cell viability was assessed by trypan blue dye exclusion (Sigma) and was always high (> 95%).

Determination of total cell-associated u-PA activity

Total cell-associated u-PA activity (intracellular plus membrane-bound) was measured in stimulated and resting (non-stimulated) neutrophils. Preliminary experiments indicated that optimal stimulation of neutrophils was achieved after treatment with 81 μM-PMA for 30 min at 37 °C. In these experiments, neutrophils were treated with different concentrations of PMA (0–162 μM) for various incubation times (0–180 min). Incubation of neutrophils with PMA for periods longer than 60 min produced cytotoxic effects. A temperature of 37 °C is optimal for maintaining neutrophils *in vitro*. Neutrophils maintained at 33 ° or 39 °C had reduced viability.

Neutrophils (2×10^6/ml) were resuspended in 500 μl of Hanks balanced salt solution (HBSS; Sigma) containing 20 mM-HEPES (Sigma) with or without 81 μM-PMA (Sigma). After incubation for 30 min at 37 °C, neutrophils were washed three times with HBSS. Neutrophils were then lysed by addition of 500 μl 1 mM-sodium bicarbonate with or without Triton X-100 (0·2%, v/v), centrifuged at 12000 ***g*** for 3 min, aliquoted, and stored at −80 °C. Activity of u-PA in aliquots of lysed neutrophils was determined by the procedure that has been used for several cell types and is described by Cheli *et al*. (1999).

The assay utilizes the enzymatically active u-PA present within lysed neutrophils to convert exogenously supplied plasminogen to active plasmin. Plasmin, so produced, is subsequently allowed to attack the chromogenic substrate Val-Leu-Lys-*p*-nitroaniline adjacent to lysine and liberate the free chromophore *p*-nitroaniline. In this system, changes in colour are directly related to plasmin concentrations, and, therefore, indirectly to u-PA activity.

Determination of membrane-bound u-PA activity

Membrane-bound u-PA is catalytically active (Plesner *et al*. 1994) and, thus, its activity can be measured in 'live' PMA-stimulated and resting neutrophils. Neutrophils (2×10^6/ml) were resuspended in 500 μl HBSS containing 20 mM-HEPES with or without 81 μM-PMA. After incubation for 30 min at 37 °C, neutrophils were washed three times with HBSS and, finally, resuspended at various cell concentrations in 250 μl 100 mM-Tris buffer, pH 8·0, containing 100 mM-NaCl, plasminogen (50 μg/ml; Sigma), and 0·6 mM-Val-Leu-Lys-*p*-nitroanilide (Sigma). The reaction mixture was incubated for up to 3 h and absorbance at 405 nm was measured at 15-min intervals using a microtiter plate reader. Rate of *p*-nitroaniline formation was calculated from the linear part of the curve for absorbance versus time. A sample without plasminogen served as a control. Preliminary experiments indicated that u-PA activity was linear for up to 3 h incubation and cells remained viable and intact throughout this period.

To eliminate the possibility that some u-PA was secreted in the medium, neutrophils from control wells were removed by centrifugation at the end of the

incubation period. No detectable activity was found in these samples, indicating that no u-PA was released during the incubation period. Therefore, any changes in colour are due to conversion of plasminogen to plasmin by catalytically active u-PA present on the cell membrane.

Determination of free u-PA binding sites on the cell membrane of neutrophils

u-PA binding sites present on the cell membrane of neutrophils may or may not be fully saturated (Plesner *et al*. 1994). Free, unoccupied u-PA binding sites can be fully saturated following incubation of neutrophils with purified u-PA. Thus, the difference of membrane-bound u-PA before and after incubation with u-PA reflects the presence of free u-PA binding sites on the cell membrane of neutrophils.

Neutrophils (2×10^6/ml) were resuspended in 500 μl HBSS containing 20 mM-HEPES with or without 81 μM-PMA. After incubation for 30 min at 37 °C, neutrophils were washed three times with HBSS and resuspended in 500 μl HBSS plus 20 mM-HEPES containing purified u-PA (Sigma; 10 Units/ml). After incubation for a further 30 min at 37 °C, neutrophils were washed three times with HBSS, and then resuspended at various cell concentrations in 250 μl 100 mM-Tris buffer, pH 8·0, for determination of the membrane-bound u-PA as described above.

A small deviation from the above method was used to ensure that incubation with purified u-PA for 30 min was sufficient to fully saturate the free u-PA binding sites on the cell membrane of neutrophils. Neutrophils, isolated as described above, were incubated with or without 81 μM-PMA in the presence of purified u-PA (10 Units/ml). Neutrophils were incubated for 0, 10, 20, 30, 60 or 180 min. Membrane-bound u-PA was determined as described above. Cell viability was assessed by trypan blue dye exclusion.

Determination of u-PA and u-PAR mRNA

Neutrophils (2×10^6/ml) were resuspended in 500 μl HBSS containing 20 mM-HEPES plus 81 μM-PMA. After incubation for 30 min at 37 °C, neutrophils were washed three times with HBSS. Neutrophils from several wells were collected, combined and total RNA was extracted using the guanidium thiocyanate isolation procedure (Chomczynski & Sacchi, 1987). Total RNA was separated by electrophoresis and then transferred to Zeta probe membranes. Membranes were hybridized with labelled bovine u-PA cDNA (Ravn *et al*. 1995) or u-PAR cDNA (Reuning *et al*. 1993). All details regarding electrophoresis, transfer, hybridization, stripping of membranes including necessary controls have been described previously (Cheli *et al*. 1999).

Protein kinase C involvement

To assess the role of protein kinase C in the regulation of u-PA induction by PMA, neutrophils (2×10^6/ml) were resuspended in 500 μl HBSS containing 20 mM-HEPES, 81 μM-PMA and various concentrations of 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride (H-7; 10–100 μM/l) or various concentrations of N-(2-quanidinoethyl)-5-isoquinoline sulphonamide hydrochloride (HA-1004; 10–100 μM/l). After incubation for 30 min at 37 °C, cells were washed, recovered and total cell-associated and membrane-bound u-PA activities were determined as described above.

In another experiment, neutrophils were stimulated with 81 μM-4-phorbol 12,13 didecanoate (PDDA) instead of PMA. PDDA belongs to the family of phorbol esters

Table 1. *Total cell-associated and membrane-bound urokinase-plasminogen activator (u-PA) activity of resting neutrophils incubated alone or with purified exogenous u-PA*†

(Values are means ± SD for 40 independent samples)

Type of activity	u-PA activity, ΔA/h
Total cell-associated	0·053 ± 0·012
Membrane-bound, no exogenous u-PA added	0·052 ± 0·015
Membrane bound, exogenous u-PA added	0·824 ± 0·098**

† Neutrophils (2×10^6/ml) were incubated alone or with exogenous purified u-PA (10 Units/ml).
** Values significantly different from those of neutrophils incubated without exogenous u-PA: $P < 0{\cdot}01$.

but does not activate PKC (Lowe *et al.* 1992). Total cell-associated and membrane-bound u-PA activity was determined as described above.

Statistical analysis

Values are reported as means and SD. Statistical significance of differences between means was determined using Student's *t* test.

RESULTS

Resting neutrophils

Low amounts of total cell-associated or membrane-bound u-PA were found in resting neutrophils (Table 1). In a limited number of samples ($n = 10$), the inclusion of a detergent (Triton X-100) to ensure that neutrophils were completely lysed following hypotonic shock, had no effect on the cell-associated u-PA activity of neutrophils. More specifically, total cell-associated u-PA activity, expressed as difference in absorbance per hour (mean ± SD) in neutrophils lysed with or without a detergent was 0·051 ± 0·017 and 0·048 ± 0·018, respectively. The majority of cell-associated u-PA appears to be present on the cell membrane (Table 1). However, incubation of neutrophils for 30 min in the presence of purified u-PA (10 IU/ml of medium/2×10^6 cells) led to a dramatic increase (16-fold, $P < 0{\cdot}01$) in membrane-bound u-PA suggesting that resting neutrophils have numerous free, unoccupied u-PA binding sites.

Whether the binding of exogenous u-PA was specific or not was examined. Addition of ATF (100 μM), the proteolytically inactive fragment of u-PA that binds to u-PAR, in the culture medium prior to addition of purified exogenous u-PA blocked the binding of u-PA to the neutrophil cell membrane (Table 2). This indicates that the binding of u-PA to membrane of neutrophils is specific.

Incubation of neutrophils for 30 min in the presence of purified u-PA was sufficient to fully saturate the free u-PA binding sites on the cell membrane of neutrophils (Table 3).

Activated neutrophils

The effect of PMA on total cell-associated and membrane-bound u-PA activity was examined (Table 4). PMA caused a dramatic increase (18-fold, $P < 0{\cdot}01$) in total cell-associated and membrane-bound u-PA but, surprisingly, caused a substantial decrease ($P < 0{\cdot}01$) in free, unoccupied u-PA binding sites. Northern blot analysis indicated that PMA caused two- to eight-fold increases in u-PA mRNA (Fig. 1) and u-PAR expression (Fig. 2) by bovine neutrophils.

Table 2. *Membrane-bound urokinase-plasminogen activator activity (u-PA) of resting neutrophils incubated with purified u-PA supplemented with various amounts of purified amino terminal fragment of u-PA (ATF)*†

(Values are means ± SD for 10 independent samples)

ATF†, μM	Membrane-bound u-PA, ΔA/h
0	0·832 ± 0·093
1	0·802 ± 0·097
10	0·379 ± 0·045**
100	0·173 ± 0·035**

† ATF is a proteolytically inactive amino terminal fragment of u-PA (residues 1–135) which blocks binding of u-PA to the cell membrane. Neutrophils (2×10^6/ml) were incubated with exogenous purified u-PA (10 Units/ml) and various amounts (0–100 μM) of ATF.

** Values are significantly different from those of neutrophils incubated with exogenous u-PA and without ATF addition: $P < 0{\cdot}01$.

Table 3. *Membrane-bound urokinase-plasminogen activator (u-PA) activity of resting neutrophils incubated with purified u-PA for various times*†

(Values are means ± SD for 10 independent samples)

Incubation time, min	Membrane-bound u-PA, ΔA/h
0	0·049 ± 0·023[a]
10	0·482 ± 0·097[b]
20	0·653 ± 0·123[c]
30	0·836 ± 0·167[d]
60	0·807 ± 0·160[d]
180	0·657 ± 0·087[c]

† Neutrophils (2×10^6/ml) were incubated with exogenous purified u-PA (10 Units/ml).

[a,b,c,d] Values with different superscript letters are significantly different: $P < 0{\cdot}05$.

Table 4. *Effect of phorbol myristate acetate (PMA) on total cell-associated, membrane-bound urokinase plasminogen activator (u-PA) activity and free-unoccupied u-PA binding sites on the plasma membrane of bovine neutrophils*

(Values are means ± SD for 40 independent samples)

	u-PA activity, ΔA/h		
PMA, μmol/l	Total cell-associated	Membrane-bound	Free u-PA binding sites
0	0·052 ± 0·015	0·050 ± 0·018	0·996 ± 0·097
81	0·922 ± 0·102**	0·890 ± 0·092**	0·402 ± 0·045**

** Values were significantly different from those of neutrophils incubated without PMA added: $P < 0{\cdot}01$.

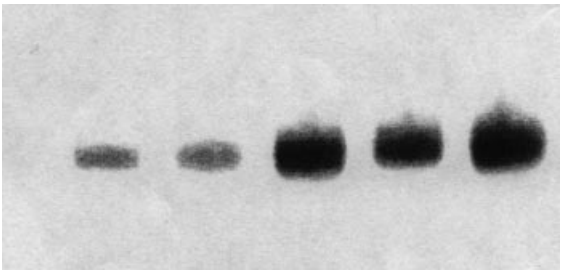

Fig. 1. Northern blot analysis of urokinase-plasminogen activator (u-PA) mRNA from resting and phorbol myristate acetate (PMA) activated neutrophils. Lanes 1–3 and 4–6 were loaded with RNA isolated from resting and PMA-activated neutrophils, respectively.

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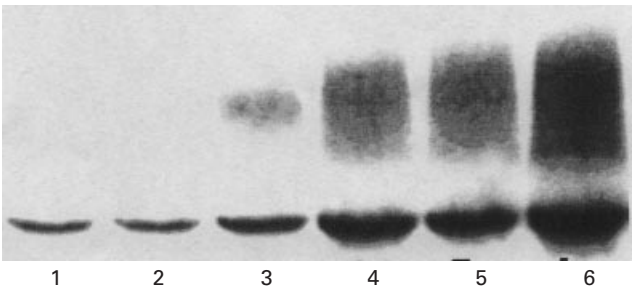

Fig. 2. Northern blot analysis of urokinase-plasminogen activator receptor (u-PAR) mRNA from resting and phorbol myristate acetate (PMA) activated neutrophils. Lanes 1–3 and 4–6 were loaded with RNA isolated from resting and PMA-activated neutrophils, respectively.

Table 5. *Effect of various protein kinase inhibitors on total cell-associated urokinase plasminogen activator (u-PA) activity of resting neutrophils and neutrophils activated by phorbol myristate acetate (PMA, 81 μmol/l) bovine neutrophils*

(Values are means ± SD for 10 independent samples)

	u-PA activity, ΔA/h	
	Resting neutrophils	Activated neutrophils
Control	0·115 ± 0·017	0·852 ± 0·095
+ H7 (10 μmol/l)	0·117 ± 0·019	0·314 ± 0·087**
+ H7 (100 μmol/l)	0·096 ± 0·011	0·164 ± 0·017**
+ HA1004 (10 μmol/l)	0·061 ± 0·010	0·792 ± 0·108
+ HA1004 (100 μmol/l)	0·057 ± 0·015	0·778 ± 0·095

H7: 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride.
HA-1004: (2-quanidinoethyl)-5-isoquinoline sulphonamide hydrochloride.
** Values were significantly different from those of the control: $P < 0·01$.

Table 6. *Comparison of the effects of phorbol myristate acetate (PMA) and 4α-phorbol 12,13 didecanoate (PDDA) (81 μmol/l) on total cell-associated and free u-PA binding sites on the plasma membrane of bovine neutrophils*

(Values are means ± SD for 10 independent samples)

	u-PA activity, ΔA/h	
	Total cell-associated	Free u-PA binding sites
Control	0·102 ± 0·012	0·876 ± 0·157
+ PMA	0·919 ± 0·126**	0·327 ± 0·067**
+ PDDA	0·129 ± 0·022	0·923 ± 0·177

** Values were significantly different from those of the control: $P < 0·01$.

PKC involvement

The effect of two different protein kinase inhibitors on total cell-associated u-PA activity of neutrophils activated by PMA was examined. Addition of H7, which is a potent inhibitor of both PKA and PKC, completely abolished the effect of PMA on total cell-associated u-PA activity (Table 5). Addition of HA1004, which is a potent PKA but a weak PKC inhibitor, had no effect on total cell-associated u-PA activity.

To examine further the role of PKC, the effect of PDDA, a phorbol ester that does not activate PKC, on total cell-associated u-PA activity of neutrophils was examined. Addition of PDDA had no effect on total cell-associated u-PA activity of neutrophils (Table 6).

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discussion

The first finding from the present study was that resting neutrophils produce very low amounts of u-PA. Furthermore, the majority of the cell-associated u-PA in resting neutrophils was found bound to the cell membrane. Interestingly, resting neutrophils have numerous free, unoccupied u-PA binding sites on their cell membrane. This conclusion is supported by results showing a dramatic increase in membrane-bound u-PA following incubation of resting neutrophils with purified exogenous u-PA. In fact, only 6% of the total u-PA binding sites on the cell membrane of resting neutrophils are occupied with u-PA. These results are consistent with those of Plesner *et al.* (1994) who showed a large excess of unoccupied u-PA binding sites on the plasma membrane of resting human neutrophils.

Binding of u-PA to the cell membrane of resting neutrophils is specific. This conclusion is supported by results showing that addition of ATF together with exogenous u-PA in the culture medium of resting neutrophils competed and eventually blocked binding of u-PA to the cell membrane. The u-PA molecule consists of a number of functional domains: 1) the EGF–like domain which is structurally similar to the receptor-binding region of EGF, 2) one, so-called, kringle structure, and 3) the carboxy-terminal region, which contains the active site of u-PA. The EGF-like domain and the kringle structure make up the ATF region of the u-PA molecule. ATF mediates the binding of u-PA to u-PAR and lacks enzymatic activity (Politis, 1996).

Low u-PA activity detected intracellularly in resting neutrophils is somewhat surprising. This finding suggests that bovine neutrophils produce low amounts of u-PA and they lack intracellular stores of u-PA. In contrast, resting human neutrophils produce high amounts of u-PA which is stored intracellularly; the stored u-PA is translocated to the cell membrane following stimulation with PMA (Heiple & Ossowski, 1986; Plesner *et al.* 1994). We consider it possible that bovine neutrophils, in a manner similar to their human counterparts, have high amounts of intracellular u-PA. However, bovine neutrophils were not completely lysed following hypotonic shock and, thus u-PA was not freed from the putative intracellular sources. Two pieces of evidence argue against this possibility. First, inclusion of a detergent (Triton X-100) to ensure that bovine neutrophils were completely lysed had no effect on total cell-associated u-PA of neutrophils. Second, the low intracellular u-PA activity appears to be in line with minimal u-PA mRNA levels detected in resting neutrophils. Transcription of the u-PA gene is a prerequisite for u-PA protein production.

The second finding from the present study was that PMA caused increases in the activity of total cell-associated, membrane-bound u-PA and u-PA mRNA. More specifically, PMA caused on average 18-, 17·8- and 4-fold increases in total cell-associated u-PA, membrane-bound u-PA and u-PA mRNA, respectively. In a similar fashion to that seen with resting neutrophils, the majority of the cell-associated u-PA is found bound to the cell membrane. On the other hand, approximately 69% of the total u-PA binding sites of PMA-activated neutrophils are occupied with u-PA. Despite the increase in total (occupied plus free) u-PA binding sites of activated neutrophils compared with those of resting neutrophils (Tables 1 and 4), the main effect of PMA is an increase in production of u-PA by activated neutrophils (Table 4); the u-PA so produced then occupies the available free u-PA binding sites leading to a decrease in the free, unoccupied u-PA binding sites on the cell membrane of neutrophils.

Consistent with our findings, others reported that PMA-activated human neutrophils had approximately 12-fold more membrane-bound u-PA than their resting counterparts (Heiple & Ossowski, 1986). Plesner *et al.* (1994) reported a dramatic increase in membrane-bound u-PA and total u-PA binding sites on the membrane of human neutrophils. However, the majority (90%) of total u-PA binding sites were free, unoccupied. Thus, PMA caused similar increases in both membrane-bound u-PA and total u-PA binding sites on the cell membrane of human neutrophils. Thus, the ratio of free to total u-PA binding sites remained unaltered.

Of greater biological significance is the observation that activated bovine neutrophils, in similar fashion to their human counterparts, have more membrane-bound u-PA. This is because, during recruitment, neutrophils respond to chemoattractants and traverse matrix barriers. It is conceivable that u-PA bound to the neutrophil cell receptor would promote proteolysis, thus making it easier for the activated neutrophils to reach the inflamed tissue.

One observation that deserves some discussion is that PMA caused two- to eight-fold increases in u-PA and u-PAR mRNA and 18-fold increases in total cell-associated and membrane-bound u-PA. This indicates that PMA may have affected u-PA and u-PAR mRNA stability or u-PA and u-PAR protein translation.

The third finding from the present study was that the effects of PMA on total cell-associated and membrane-bound u-PA are mediated through activation of PKC. This conclusion is supported by two observations. First, H7 which is a potent, but non-specific inhibitor of PKC, which also inhibits cAMP- and cGMP-dependent protein kinases (Hidaka *et al.* 1984), blocked the effect of PMA on total cell-associated u-PA activity. Furthermore, addition of HA1004, which is a potent PKA inhibitor but a weak PKC inhibitor (Asano & Hidaka, 1984), had no effect on total cell-associated u-PA activity. These data, taken collectively, indicate that PKC mediates at least part of the effects of PMA on the u-PA system. Second, addition of PDDA, instead of PMA, to the neutrophil cell culture, a phorbol ester that does not activate PKC (Lowe *et al.* 1992), had no effect on total cell-associated u-PA activity of neutrophils.

Others reported that effects of PMA on bovine neutrophils depend upon activation of PKC (Dore *et al.* 1992). Allard *et al.* (1999) showed that PMA increased respiratory burst in bovine neutrophils and this increase required activation of PKC.

In summary, our results demonstrate that PMA caused increases in production of u-PA and u-PAR by neutrophils at both the mRNA and protein levels. The u-PA so produced then occupies available free u-PA binding sites on the cell membrane of neutrophils. The demonstration of increased u-PA and u-PAR in neutrophils activated by PMA strengthens the suggestion that cell-associated protease activity is an important element in the cohort of functions altered in cells capable of migration. Understanding the fundamental elements of the u-PA/u-PAR system is an absolute prerequisite prior to devising methods to upregulate u-PAR expression in bovine neutrophils, thus facilitating neutrophil migration.

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