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EVIDENCE FOR ECTO-PROTEIN KINASE ACTIVITY ON THE SURFACE OF HUMAN NEUTROPHILS[+]

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Recent studies have suggested a possible role for extracellular ATP in neutrophil function. This report provides evidence for the existence of an ecto-protein kinase activity on the surface of human neutrophils capable of phosphorylating intrinsic cell membrane proteins as well as exogenous proteins. Addition of extracellular [γ-^{32}P]ATP to neutrophils resulted in rapid incorporation of ^{32}P into cellular proteins that were sensitive to trypsin. The ability of adherent cells to phosphorylate the exogenous substrate casein, while no protein kinase activity was released into the supernate, provided further evidence for a cell surface associated protein kinase activity.

Protein phosphorylation is an important regulatory mechanism in many cells, including human neutrophils (1-6). While most studies of protein phosphorylation have focused on intracellular protein kinases (2,4), recent studies have provided evidence for the existence of ecto-protein kinase activity on the surface of several different types of cells (7-11). Other recent studies suggesting effects of extracellular ATP on human neutrophil or murine macrophage function (12-15) led us to search for ecto-protein kinase activity on human neutrophils. This report provides evidence for the existence of an ecto-protein kinase on the surface of human neutrophils, identifies several endogenous substrates for its activity, and characterizes the kinetics and divalent cation requirements of this kinase activity.

METHODS

Ecto-kinase assay. Normal human neutrophils, prepared from heparinized venous blood by dextran sedimentation at 1 x g followed by Ficoll-Hypaque

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Abbreviations:
NH - buffer containing 140 mM NaCl and 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.4; HBSS - Hanks' balanced salt solution, pH 7.4; SDS-PAGE - $NaDodSO_4$ - polyacrylamide gel electrophoresis

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centrifugation as previously described (16), were suspended at the indicated concentration in 140 mM NaCl and 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.4 (NH). Differential cell counts routinely revealed > 95% neutrophils. Protein kinase activity at the cell surface was assayed by adding 20 µl of NH containing various ions, as indicated, to 40 µl of NH containing 1 x 10^6 neutrophils and 10 µCi of [γ-^{32}P]ATP (specific activity 4500 Ci/mmol, ICN) in Eppendorf tubes, and incubating the mixture at 37°C for the indicated time. Diisopropylfluorophosphate (DFP) (Sigma), unless otherwise indicated, was then added (final concentration 10 mM) and the samples were incubated for 2 min at 23°C. The mixture was then centrifuged at 13,000 x g for 2 min at 23°C, the supernatants were removed and added to twice concentrated sample buffer (16) and the cells were resuspended in 70 µl of sample buffer. Samples were immediately incubated for 2 min at 100°C and analyzed by $NaDodSO_4$-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were stained, dried, and examined by autoradiography using Dupont Cronex film.

In some cases, cells were allowed to adhere before assaying ecto-kinase activity. In these cases, 1 - 10 x 10^5 cells in 50 µl of Hanks' balanced salt solution, pH 7.4 (HBSS) were allowed to adhere for 30 min at 37°C to wells of a 96 well microtiter plate in 5% CO_2. The buffer was then aspirated and the adherent cells were washed twice with 200 µl of NH at 23°C and 20 µl of NH was added to each well. Sixty µl of NH containing 10 µCi of [γ-^{32}P]ATP and various ions as indicated were then added and the mixture was incubated for 10 min at 37°C. After treatment with DFP as described above, the supernatant was removed and analyzed as above. Sample buffer (70 µl) was then added to each well and after 1 min the solubilized cell extract was removed, incubated at 100°C for 2 min and analyzed by SDS-PAGE as above. In some cases, as indicated, 10 µg of casein (Sigma) were added to the reaction mixture and ^{32}P incorporation was determined as described above.

Radiolabelling with $H_3{}^{32}PO_4$. Experiments identical to those described above were performed using 10^3 or 100 µCi of $H_3{}^{32}PO_4$ (carrier free, ICN) in place of [γ-^{32}P]ATP. In some cases, equivalent concentrations of unlabelled ATP (Sigma) (4×10^{-13} M) were also added.

Cell Surface Trypsinization. In some cases, before or after radiolabelling, 10^6 cells were suspended in 20 µl of NH containing 0.01% TLCK-trypsin (Sigma) in Eppendorf tubes for 10 min at 37°C to remove trypsin sensitive cell-surface proteins. Cells were recovered after trypsinization by adding 1 ml of NH and centrifuging at 13,000 x g for 2 min at 23°C. Cells remained >95% viable after trypsin treatment as determined by dye exclusion.

RESULTS

Divalent cation dependence of protein phosphorylation. The addition of [γ-^{32}P]ATP to live human neutrophils in the presence of 1.8 mM Ca^{2+}, 0.8 mM Mg^{2+}, and 1 mM Mn^{2+} resulted in the incorporation of ^{32}P into a variety of proteins (Fig 1, lanes A and B). Similar ^{32}P labelling of proteins occurred in the presence of 1 mM Mn^{2+} without Ca^{2+} or Mg^{2+} (Fig 1, lanes C and D). The addition of the phosphatase inhibitors Na_3VO_4 and Na_2MoO_4 (lanes B and D) resulted in much more detectable ^{32}P-labelled protein than when the phosphatase inhibitors were not present (lanes A and C), but did not alter the electrophoretic pattern of the phosphoproteins. Therefore, 200 µM Na_3VO_4 and 200 µM Na_2MoO_4 were added to all radiolabelling reactions described here unless otherwise indicated. No protein radiolabelling was detected in the absence of divalent cations (lane E). Little protein radiolabelling was detected in the presence of 1.8 mM Ca^{2+} alone (Lane I). Much less radiolabelling was observed with 0.8 mM Mg^{2+} alone (lane J), or 1.8 mM Ca^{2+} and 0.8 mM Mg^{2+} (lane F) than

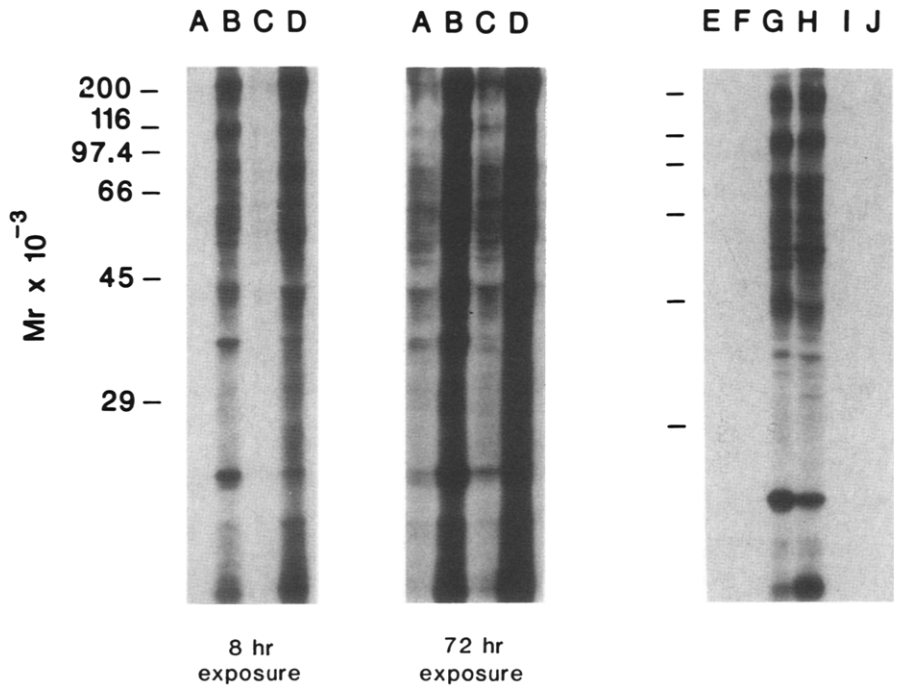

Figure 1. ^{32}P-labelling and SDS-PAGE of live human neutrophils. Cells were radiolabelled in the presence of [γ-^{32}P]ATP and the indicated concentrations of divalent cations, and analyzed by SDS-PAGE and autoradiography as described in the text. Lanes A,B,G: 1.8 mM Ca^{2+}, 0.8 mM Mg^{2+}, 1 mM Mn^{2+}; lanes C,D,H: 1 mM Mn^{2+} lane E: no divalent cations; lane F: 1.8 mM Ca^{2+} and 0.8 mM Mg^{2+}; lane I: 1.8 mM Ca^{2+}; lane J: 0.8 mM Mg^{2+}; lanes B,D,E-J, : 200 μM Na_3VO_4 and 200 μM Na_2MoO_4. Two different exposures of the autoradiograph of lanes A-D are shown to demonstrate that the phosphoprotein patterns seen in the presence and absence of Na_3VO_4 and Na_2MoO_4 are similar. Proteins used as molecular weight standards (Sigma) were: myosin heavy chain, 200,000; E. coli β-galactosidase, 116,000; phosphorylase B, 97,400; bovine serum albumin, 66,000; ovalbumin, 45,000; and carbonic anhydrase, 29,000.

was seen in the presence of 1.8 mM Ca^{2+}, 0.8 mM Mg^{2+}, and 1 mM Mn^{2+} (lane G) or 1 mM Mn^{2+} alone (lane H). Similar results were seen with adherent cells (not shown). Cell viability, as determined by trypan blue exclusion, was not changed by any of the radiolabelling reactions.

Kinetics of protein phosphorylation. Incorporation of ^{32}P into proteins by live human neutrophils following the addition of [γ-^{32}P]ATP was rapid (Fig 2). Radiolabelling was maximal by 10 min (lane C) and nearly maximal after only 1 min (lane A). The addition of unlabelled ATP caused a dose dependent decrease in protein radiolabelling, and the non-hydrolyzable ATP analogue AMP-PNP (2.5 mM) eliminated ^{32}P-labelling while the addition of 10 mM $NaPO_4$ had no effect (not shown). No protein radiolabelling was detected when cells were incubated with the same activity of $H_3{}^{32}PO_4$ (lanes F-H). This excludes the possibility that $^{32}PO_4$ released from [γ-^{32}P]ATP by ecto-ATPase activity entered the cell and was utilized to give the observed pattern of protein phosphorylation. When a 10-fold greater quantity of $H_3{}^{32}PO_4$ was added, some

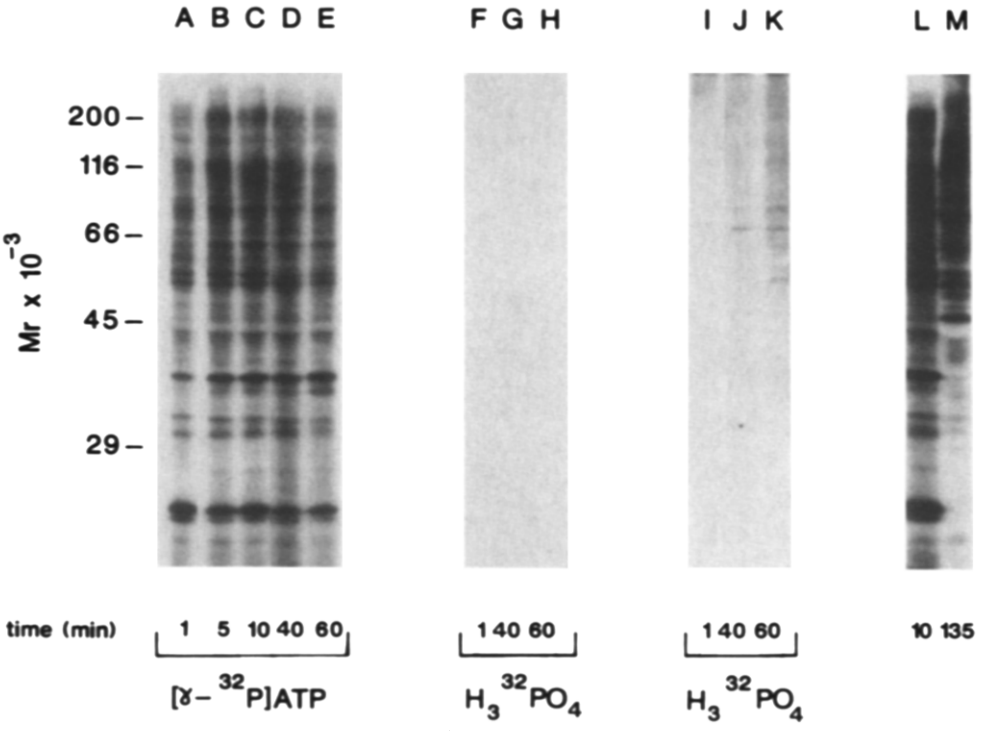

Figure 2. Kinetics of protein phosphorylation. Human neutrophils were labelled by incubation with 10 μCi [γ-^{32}P]ATP in the presence of 1 mM Mn^{2+}, 0.8 mM Mg^{2+}, 1.8 mM Ca^{2+}, 200 μM Na_3VO_4 and 200 μM Na_2MoO_4, (lanes A-E, and L) or loaded with $H_3{}^{32}PO_4$ [10 μCi, lanes F-H; 100 μCi, lanes I-K and M] for various times, as indicated, and analyzed by SDS-PAGE and autoradiography as described in the text. Lanes I-K were exposed three times as long as the other lanes.

protein radiolabelling could be detected (lanes I-K). Lanes I-K were exposed 3 times longer than the other lanes of Fig 3 to show the pattern of protein radiolabelling. Lanes L and M show ^{32}P-labelled proteins from cells labelled with [γ-^{32}P]ATP or $H_3{}^{32}PO_4$ analyzed by SDS-PAGE on the same gel for comparison. The pattern of ^{32}P-labelled proteins seen in cells labelled with [γ-^{32}P]ATP (lane L) was different from that observed from cells loaded with $H_3{}^{32}PO_4$ (lane M).

Effect of trypsin treatment on cell-surface protein phosphorylation. To confirm that the ^{32}P-labelled proteins under study were cell-surface proteins, cells were treated with trypsin before or after radiolabelling (Fig 3). When cells were incubated for 10 min in NH before radiolabelling by incubation with [γ-^{32}P]ATP, the pattern of ^{32}P-labelled proteins (Fig 3, lane B) was similar to that seen with untreated cells (lane A). In contrast, when cells were incubated with trypsin before radiolabelling (lane C), the quantity of ^{32}P-labelled proteins detected was markedly decreased. When radiolabelled cells were incubated for 10 min in NH, the pattern of ^{32}P-labelled proteins (lane D) was similar to that seen in untreated cells (lane A) and a small amount of ^{32}P-labelled proteins was detected in the supernate of the incubation reaction

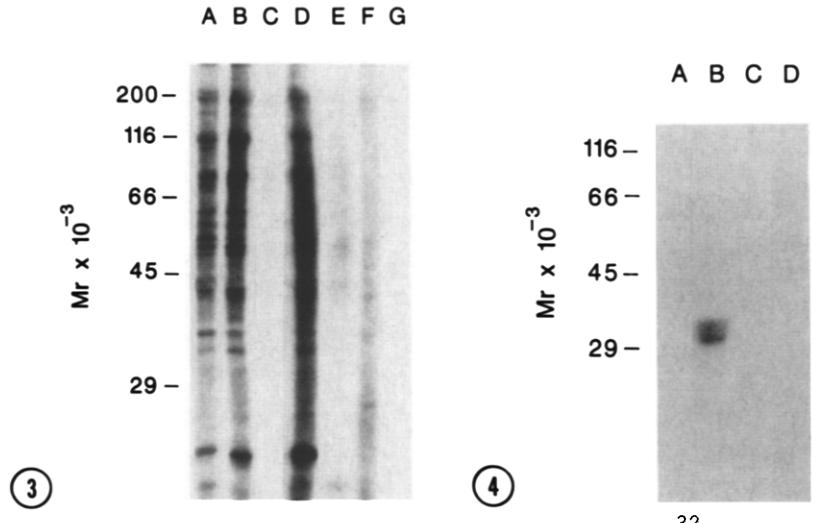

Figure 3. Trypsin treatment of neutrophils before or after ^{32}P-labelling. Cells were incubated with NaCl-HEPES (lanes B and D) or 0.01% TLCK-trypsin for 10 min at 37°C (lanes C and F) before (lanes B and C) or after (lanes D and F) radiolabelling with [γ-^{32}P]ATP in the presence of 1 mM Mn^{2+}, 0.8 mM Mg^{2+}, and 1.8 mM Ca^{2+} as described in the text. Cells incubated after radiolabelling were recovered by centrifugation, and cells and reaction supernatant were analyzed separately. Lane E, supernatant of NaCl-HEPES incubation; lane G, supernatant of trypsin incubation. Samples were analyzed as in Figure 1. Cells remained > 95% viable after trypsin treatment as determined by dye exclusion.

Figure 4. Phosphorylation of exogenous casein by live human neutrophils. Neutrophils (1.25 x 10^5/well) were allowed to adhere in HBSS to 96 well microtiter plates for 30 min at 37°C, washed, and 60 µl of NH containing 10 µCi [γ-^{32}P]ATP, 1 mM Mn^{2+}, 200 µM Na_2MoO_4, 200 µM Na_3VO_4, with (lane B) or without (lane A) 10 µg of casein, and the mixture was incubated for 10 min at 37°C. The supernate was removed and analyzed by SDS-PAGE and autoradiography as described in the text. In companion experiments, identically prepared adherent neutrophils were incubated for 10 min at 37°C in the same reaction buffer described above without [γ-^{32}P]ATP or casein and the supernatant was collected, centrifuged at 13,000 x g for 10 min and then tested for protein kinase activity (lanes C and D). Ten µg of casein and 10 µCi [γ-^{32}P]ATP were suspended in 60 µl of NH containing 1 mM Mn^{2+}, 200 µM Na_2MoO_4, and 200 µM Na_3VO_4 (lane C) or 60 µl of supernatant from adherent cells (lane D), incubated for 10 min at 37°C, and analyzed as in Figure 1.

(lane E). In contrast, incubation of radiolabelled cells with trypsin markedly decreased with the amount of ^{32}P-labelled proteins remaining cell-associated (lane F) and little radiolabelled protein was detected in the supernate of the trypsin incubation (lane G). This brief trypsin treatment did not affect cell viability as determined by dye exclusion.

Phosphorylation of exogenous proteins. To test the ability of the ecto-kinase to phosphorylate exogenous substrates, casein was added to the reaction mixture (Fig 4). Extracellular casein was rapidly phosphorylated by live human neutrophils in the presence of [γ-^{32}P]ATP (lane B) but little autophosphorylation occurred in the absence of cells (lane C). No kinase activity could be detected in the supernatant of identically treated neutrophils (lane D) using casein as a phosphate acceptor, thus confirming that the kinase activity was present on the cell surface.

DISCUSSION

While most studies of protein phosphorylation have been directed at intracellular reactions, recent studies have suggested the existence of ecto-protein kinase activity on the surface of several types of cells (7-11). Nucleotides can be released by many cell types including endothelial cells, platelets, and neurons, but the physiologic role of these nucleotides is not well defined (17,18). Other recent studies have suggested a possible role for extracellular ATP in human neutrophil function (12,14,15).

The studies reported here provide evidence for ecto-protein kinase activity on the surface of human neutrophils. Addition of extracellular [γ-^{32}P]ATP, but not $H_3{}^{32}PO_4$, to neutrophils resulted in rapid incorporation of ^{32}P into cellular proteins. In addition, the pattern of protein phosphorylation seen with $^{32}PO_4$ loading of cells was different from that seen with the addition of [γ-^{32}P]ATP. These results suggest that the protein phosphorylation seen with the addition of extracellular [γ-^{32}P]ATP was not due to uptake and utilization of $^{32}PO_4$ released by ecto-ATPase action. The sensitivity to trypsin of the proteins radiolabelled by addition of [γ-^{32}P]ATP also suggests that these proteins are located on the cell surface. The ability of adherent cells to phosphorylate the added exogenous substrate casein while no protein kinase activity was detectable in supernates from identical cell preparations using the same substrate, provided further evidence for a cell surface associated protein kinase activity.

While Mn^{2+} was the most potent divalent cation studied at supporting ecto-kinase activity (activity detected at <4 μM Mn^{2+}), significant ecto-kinase activity was also detected at physiologic concentrations of Mg^{2+} (not shown). The relationship of the observed ecto-protein kinase activity to the previously described Mg^{2+}-dependent ecto-ATPase on neutrophils (19) is not known. Stimulation of neutrophils with N-formyl-met-leu-phe or TPA did not alter ecto-kinase activity (not shown), however, the rapid kinetics of protein phosphorylation may have obscured any effects of these agents. Thus, these studies provide strong evidence for the existence of an ecto-protein kinase activity on the surface of human neutrophils capable of phosphorylating intrinsic cell membrane proteins as well as exogenous proteins. Phosphorylation of surface proteins may play a role in neutrophil function while phosphorylation of extracellular proteins may modulate their function or uptake and processing by other cells. For example, phosphorylation of extracellular lysosomal enzymes could alter their uptake by cells via the mannose-6-phosphate receptor (20). In addition, neutrophil ectokinase might phosphorylate surface proteins of other cells. While it seems likely that this kinase is physiologically important, its exact role in neutrophil function remains to be defined.

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REFERENCES

1. Andrews, P.C., and Babior, B.M. (1983) Blood 61, 333-340.
2. Nishizuka, Y. (1984) Nature 308, 693-698.
3. Segal, A.W., Heyworth, P.G., Cockcroft, S., and Barrowman, M.M. (1985) Nature 316, 547-549.
4. Sibley, D.R., Benovic, J.L., Caron, M.G., Lefkowitz, R.J. (1987) Cell 48, 913-922.
5. Changelian, P.S., and Fearon, D.T. (1986) J. Exp. Med. 163, 101-115.
6. Balazovich K. J., Smolen J. E., and Boxer L. A. (1986) J. Immunol. 137, 1665-1673.
7. Ehrlich, Y. H., Davis, T. B., Bock, E., Kornecki E., and Lenox R. H. (1986) Nature 320, 67-70.
8. Remold-O'Donnell, E. (1978) J. Exp. Med. 148, 1099-1104.
9. Kubler, D., Pyerin, W., Burow, E., and Kinzel V. (1983) Proc. Natl. Acad. Sci. 80, 4021-4025.
10. Chiang, T. M., Kang, E. S., and Kang A. H. (1979) Arch. Biochem. Biophys. 195, 518-525.
11. Haldar, S., and Majumder G. C. (1986) Biochim Biophys. Acta. 887, 291-303.
12. Freyer, D. R., Boxer, L. A., Axtell, R. A., and Todd R. F. III. (1987) Clin. Res. 35, 890A.
13. Sung, S. J., Young, J. D., Origlio, A. M., Heiple, J. M., Kaback, H. R., and Silverstein, S. C. (1985) J. Biol Chem. 260, 13442-13449.
14. Melloni, E., Pontremoli, S., Salamino, F., Sparatore, B., Michetti, M., Sacco, O., and Horecker, B.L. (1986) J. Biol. Chem. 261, 11437-11439.
15. Cameron, D. J. (1985) Immunol. Immunopathol. 37, 230-235.
16. Skubitz, K. M., and Snook, R. W., II, (1987) J. Immunol. 139, 1631-1639.
17. Gordon, J. L. (1986) Biochem. J. 233, 309-319.
18. Pearson, J. D., Gordon, J. L., (1985) Ann. Rev. Physiol. 47, 617-627.
19. Smolen, J. E., and Weissmann, G. (1978) Biochim. Biophys. Acta. 512, 525-538.
20. Shepherd, V., Schlesinger, P., and Stahl, P. (1983) Curr. Top. Membr. Transp. 18, 317-338.