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HUMAN NEUTROPHIL PHOSPHODIESTERASE
Calmodulin Insensitivity and Other Properties

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Abstract—Extracts of human neutrophils were examined for phosphodiesterase activity using a radiochemical assay. As reported by other investigators, both high- and low-K_m forms of the enzyme were found. Although calmodulin could be measured in these extracts, human neutrophil phosphodiesterase proved not to be calmodulin dependent. Activity of the neutrophil phosphodiesterase was also not altered by physiologic concentrations of indomethacin, *p*-bromophenacyl bromide, eicosatetraenoic acid, or eicosatetraynoic acid, all inhibitors of arachidonic acid metabolism. These results are relevant to stimulus–secretion coupling in neutrophils, wherein calmodulin-dependent reactions play a vital role.

INTRODUCTION

One of the earliest events in neutrophil activation is a transient increment in cAMP levels (1, 2). While the precise role of cyclic nucleotides in stimulus–response coupling remains to be elucidated (1–3), the promptness of the cAMP response and its potential as a second messenger have made it the subject of intensive investigation. On the other hand, the enzymes regulating cyclic nucleotide levels in these cells, namely adenylate (or guanylate) cyclase and phosphodiesterase, have received scant attention. The most recent work in this field is by Smith and Peters (4), who reported that human neutrophil phosphodiesterase is cytosolic and has both high- and low-K_m forms.

While the role of cyclic nucleotides in stimulus–secretion coupling in neutrophils is unclear, that of intracellular calcium is not. The mobilization of membrane-bound calcium (5–8), with attendant rises in intracellular free calcium (9, 10) and activation of calmodulin-dependent reactions (8, 11) constitutes a widely held concept of stimulus–response coupling for these

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cells. Indeed, neutrophil responsiveness can be completely blocked by antagonists of intracellular calcium and calmodulin (8, 11). Since many forms of phosphodiesterase in other tissues are calmodulin activatable (12–14), it was of interest to see if calcium mobilization and calmodulin could affect phosphodiesterase in these cells, thereby potentially modulating cyclic nucleotide metabolism. In essence, we found that human neutrophil phosphodiesterase was not affected by calmodulin or its antagonists, suggesting that regulation of enzymatic activity was accomplished by some other mechanism(s).

MATERIALS AND METHODS

Reagents. Calmodulin (phosphodiesterase activator), activator-deficient phosphodiesterase, 5′-nucleotidase, *p*-bromophenacyl bromide, cAMP, 5′-AMP, and adenosine were purchased from Sigma Chemical Company, St. Louis, Missouri. TMB-8 was a gift from Robert J. Smith (Upjohn Company), and W-7 was a generous gift of Hiroyoshi Hidaka (Mie University School of Medicine). ETYA and ETI were generously provided by Aaron Marcus.

Preparation of Cell Suspensions. Purified preparations of neutrophils were isolated from heparinized (10 units/ml) venous blood by means of Hypaque-Ficoll gradients (15), followed by standard techniques of dextran sedimentation and hypotonic lysis of erythrocytes (16).

Phosphodiesterase Assay. Pellets of human neutrophils were resuspended in an equal volume of "assay buffer," consisting of 40 mM Tris HCl, pH 8.0, 5 mM $MgCl_2$, and 1 mM dithiothreitol. This suspension was sonicated and then centrifuged for 15 min at 15,000*g*. The resulting supernatant, hereafter referred to as "extract," was harvested and employed as a source of endogenous neutrophil phosphodiesterase. The assay procedure was a minor modification of that published by Thompson et al. (17). In essence, 20 μl of extract was incubated for 1 h at 37° in 300 μl of assay buffer containing 300 μM $CaCl_2$ and 1 mM [^{3}H]cAMP (0.5 μCi/ml; New England Nuclear). The incubation was stopped by placing the tubes in a boiling-water bath for 3 min. After cooling, 50 mU of 5′-nucleotidase was added and incubation proceeded for 10 min more at 37°; this step converted 5′-AMP, produced from cAMP by phosphodiesterase, to adenosine. Unreacted cAMP was then removed by addition of 1 ml of a 2 : 1 slurry of anion exchange resin (Biorad AGI-X2). After equilibration for 15 min at room temperature, the resin was centrifuged down and aliquots of the supernatant were taken for determination of [^{3}H]adenosine by scintillation counting. For each experiment, controls were run to determine the extent of trapping of [^{3}H]cAMP and [^{3}H]adenosine by the anion-exchange resin and appropriate corrections were applied to the calculations.

Determination of Calmodulin Activity. Calmodulin present in the neutrophil extracts was most accurately determined by means of radioimmunoassay (CAABCO, Houston, Texas). Its biologic activity was assayed as follows. Extracts were boiled for 3 min to inactivate endogenous phosphodiesterase and other enzymes. Flocculent material was removed by centrifugation. Calmodulin activity was assayed in the system described above, using 1 mU of commercially available activator-dependent phosphodiesterase. Calmodulin present in the boiled extract thus increased the measured activity of the exogenous phosphodiesterase. Routine controls, applied during each experiment, included removal of calcium by EGTA and addition of the calmodulin antagonist trifluoperazine.

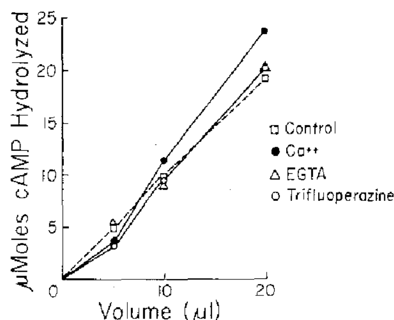

Fig. 1. The phosphodiesterase activity in various volumes of neutrophil extract was measured. The assays were performed in the presence of 0.3 mM $CaCl_2$ (Ca^{2+}), 0.4 mM EGTA, 30 μM trifluoperazine, or in the absence of these agents (control).

RESULTS

Soluble extracts of human neutrophils contained phosphodiesterase activity which was readily measured by the two-step assay system described in Materials and Methods. Analysis of the kinetics of enzymatic activity revealed two forms of the enzyme, having values for apparent K_m of 920 ± 440 μM ($N = 8$) and 20 ± 9 μM ($N = 10$); these values agree closely with those reported by Smith and Peters (4). Figure 1 shows that high-K_m phosphodiesterase activity was linear with respect to extract volume assayed, as expected. However, activity of the enzyme from neutrophils was not altered by the presence or absence of calcium or the presence of the calmodulin inhibitor trifluoperazine. Thus, it appeared the neutrophil phosphodiesterase was insensitive to calmodulin.

Figures 2 and 3 show attempts to activate neutrophil phosphodiesterase by the direct addition of commercially available calmodulin in the presence of calcium. As can be seen, calmodulin had no significant effect upon the kinetic parameters of either the high-K_m (Figure 2) or low-K_m (Figure 3) form of the enzyme. Furthermore, high-K_m neutrophil phosphodiesterase was not affected by the calmodulin inhibitor W-7, the intracellular calcium antagonist TMB-8, or EGTA (Figure 4). These results were not due to any inherent deficiencies in the assay system since activation of exogenous phosphodiesterase by either commercial or neutrophil calmodulin was blocked by EGTA, W-7, or trifluoperazine (not shown). These results were also not due to a lack of calmodulin in the neutrophil extracts; calmodulin was detected by radioimmunoassay at a concentration of 9 pmol/10^6 cells, a value in accord with that reported by Chafouleas et al (18). Biologic activity of endogenous

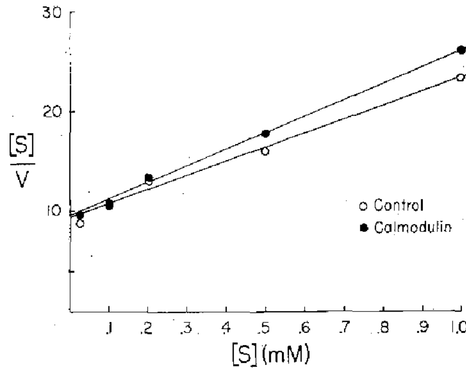

Fig. 2. Extracts from human neutrophils were assayed for phosphodiesterase activity in the presence of various concentrations of substrate. For the indicated data points, calmodulin (200 mU) was also present. The results are from a single representative experiment and provide values from apparent K_m of 593 μM (control) and 585 μM (calmodulin).

calmodulin was also demonstrated by its ability to activate susceptible commercial phosphodiesterase.

Figure 5 shows the effect of several inhibitors of arachidonic acid metabolism on neutrophil phosphodiesterase. At physiologic concentrations (10–50 μM), indomethacin (a cyclooxygenase inhibitor), BPB (a phospholipase A2 inhibitor), and ETYA and ETI (both combined inhibitors of lipoxygenase and cyclooxygenase) all had little or no effect upon the enzyme. However, at higher concentrations, indomethacin, ETYA, and ETI all significantly inhibited phosphodiesterase activity.

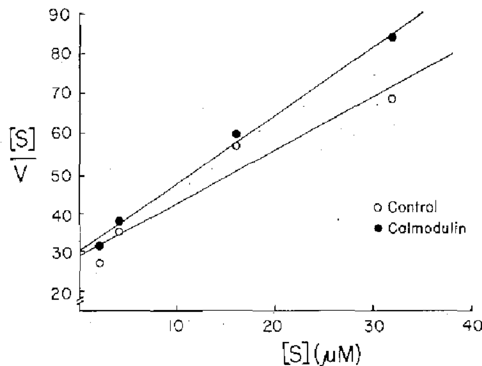

Fig. 3. Extracts from human neutrophils were assayed for phosphodiesterase activity in the presence of various concentrations of substrate. For the indicated data points, calmodulin (200 mU) was also present. The results are from a single representative experiment and provide values for apparent K_m of 22 μM (control) and 18 μM (calmodulin).

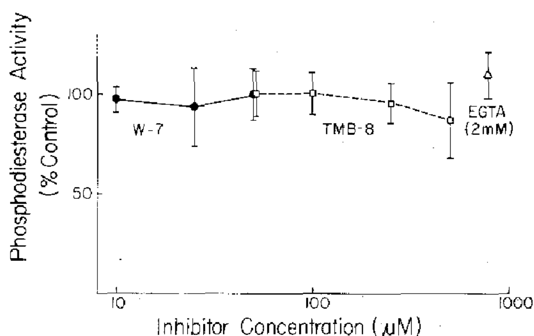

Fig. 4. Extracts from human neutrophils were assayed for high-K_m phosphodiesterase activity in the presence of the indicated concentrations of inhibitors. The results represent the means ($\pm$ *SD*; $N = 4$–6) of the percentages of control phosphodiesterase samples, to which no inhibitors were added.

DISCUSSION

In spite of the potential importance of cyclic nucleotides in modulating stimulus–secretion coupling in neutrophils, the enzymes involved in cyclic nucleotide metabolism have not been the subject of intensive investigation. With respect to human neutrophil phosphodiesterase, Smith and Peters (4) reported that it had a cytosolic localization and was found in high- and low-K_m forms. Our use of supernatant extracts from neutrophils as a source of phosphodiesterase activity is consistent with a cytoplasmic localization for this enzyme. Furthermore, our values for K_m agree with those published by Smith and Peters (4).

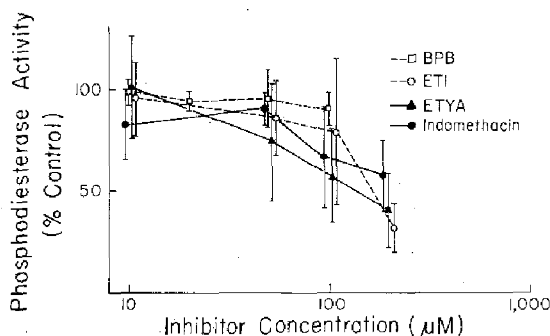

Fig. 5. Extracts from human neutrophils were assayed for high-K_m phosphodiesterase activity in the presence of the indicated concentrations of inhibitors. The results represent the means ($\pm$ *SD*; $N = 4$–6) of the percentages of control phosphodiesterase samples, to which no inhibitors were added.

Of considerable interest was our observation that neutrophil phosphodiesterase was not affected by calmodulin, calmodulin antagonists, calcium, or calcium antagonists. Thus, unlike phosphodiesterases from a variety of other cell types (12–14), this enzyme does not appear to regulated by calcium or calmodulin. While insensitivity to calmodulin is not unknown for other phosphodiesterases (12), it is, apparently, relatively rare. Since it is known that stimulation of neutrophils involves increases in cytosolic free calcium (either as a result of intracellular calcium mobilization or of influxes from the medium) as well as obligatory calmodulin-dependent reactions (5–11), this finding is significant. One ramification of this is that the recovery of baseline levels of cAMP following stimulation (1–3) is not due to calmodulin activation of phosphodiesterase. These studies thus emphasize the need for examining calmodulin requirements, if any, of neutrophil adenylate cyclase.

In view of their wide use in neutrophil research, inhibitors of lipid metabolism were employed to look for nonspecific inhibition of a neutrophil enzyme. As expected, indomethacin, BPB, ETYA, and ETI were without effect at normal physiologic concentrations (10–30 μM). However, at higher concentrations, inhibition of phosphodiesterase activity occurred, a result which is most likely nonspecific. Thus, these results emphasize the importance of using physiologic concentrations of these inhibitors and provide an example of an enzymic activity which can be nonspecifically inhibited.

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