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# CALCIUM-DEPENDENT ACTIVATION OF HUMAN NEUTROPHILS BY A SYNTHETIC IONOPHORE

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Summary: Synthetic block copolymers composed of polyoxyethylene and polyoxypropylene have been demonstrated to possess ionophore activity selective for monovalent cations and to cause histamine release from mouse mast cells and human basophils. We now report calcium-dependent release of granule contents from human neutrophils by the most active of these agents, TI30R2. At a concentration of 100 ug/ml (12.5 uM), net lysozyme release ranged from 17-40% after 30 minutes incubation at 37°. Lysozyme release was dose-dependent over stimulus concentrations of 5-50 ug/ml (0.625-6.25 uM). Release was dependent upon the presence of extracellular calcium. TI30R2 did not induce the release of superoxide anions over 30 minutes of incubation. As TI30R2 induces sodium influx into cells, it is likely that a depolarizing influx of sodium ions in the presence of extracellular calcium constitutes a sufficient signal for granule release but not superoxide production by human neutrophils. 

Activation of human neutrophils by a variety of stimuli results in the functional responses of chemotaxis, adherence, a non-mitochondrial oxidative burst, granule exocytosis, and phagocytosis (1). The complex sequence of events leading to the activation remains incompletely understood. One approach to investigate mechanisms involved in neutrophil activation sequences is to compare the optimal conditions for responses of neutrophils to stimuli which act by different pathways. The importance of calcium ions in neutrophil activation has been known for a number of years. The presence of extracellular calcium ions is required for optimal neutrophil activation by many stimuli. Similarly, neutrophil activation by many agonists is associated with a rapid influx of $Ca^{++}$ into the cells and a rapid rise in intracellular calcium which is only partially dependent on extracellular $Ca^{++}$ (1).

Recent evidence indicates that sodium ions have a role in the increase in intracellular calcium seen upon neutrophil activation. This is postulated to be mediated by a membrane-associated Na-Ca exchanger (2). A similar

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mechanism is felt to underlie the inotropic effects of cardiac glycosides (3), and the activation of other excitable cells (4). Na-Ca exchange is associated with neutrophil activation by certain agonists (2), and optimal neutrophil activation requires the presence of extracellular sodium (5). However, the observation that the sodium ionophore monensin induces partial degranulation without inducing an oxidative burst (5) indicates that additional activation pathways exist.

T130R2 is the most potent of a class of block polymers which exhibit ionophore activity for monovalent cations (6). The ability of T130R2 and its related copolymers to act as ionophores was associated with its ability to induce an inflammatory reaction in mice and the in vitro release of histamine from mast cells (7) and basophils (8). Unlike monensin and the divalent-selective carboxylic ionophore A23187, T130R2 is a neutral compound whose activity can be measured by charge flow across synthetic bilayers (6). Neutral ionophores can induce large perturbations of transmembrane potential (9). Such is not the case for charged ionophores, the action of which involves a mandatory bidirectional movement of charge. In this study we investigated the ability of T130R2 to promote neutrophil granule exocytosis and oxidative burst.

## MATERIALS AND METHODS

Materials: T130R2 was manufactured by BASF Wyandotte, Wyandotte MI and obtained through CytR$_x$ Corporation, Atlanta GA. A 1% stock solution was prepared in water and stored in aliquots at -70° C. Working solutions were made fresh prior to each experiment and kept on ice until just prior to use as previously described (7,8). Cytochrome C, superoxide dismutase, EGTA, Triton X-100, and Micrococcus Lysodeikticus were obtained from Sigma Chemical Co., St. Louis, MO.

Cells: Human neutrophils were isolated from whole blood anticoagulated with 10% acid-citrate-dextrose as previously described (10) and were suspended in buffer containing 134 mM NaCl, 8 mM KCl, 20 mM HEPES, and 1 mg/ml glucose, adjusted to pH 7.4 with NaOH. To assess release of neutrophil granules, cell suspensions (5 x $10^6$ cells/ml) warmed to 37° C for five minutes were incubated an additional five minutes with 5 ug cytochalasin B per ml of cells. Next, 0.5 ml aliquots of cells were added to an equal volume of buffer containing test agents which had been prewarmed for 5 minutes in polypropylene tubes. Following incubation the cells were placed on ice and sedimented at 1600 x g for 5 minutes and the supernatants saved.

Superoxide Release: The release of superoxide was determined by the superoxide dismutase inhibitable reduction of cytochrome C as previously described (11).

Neutrophil Granule Release: Lysozyme and LDH release were determined as previously described (10). Controls indicated no interference in the assays by the copolymer. Data are presented as the percent of net enzyme released (i.e. the percent enzyme release in the presence of the test substance minus the percent release in the absence of that substance), and for each experiment were determined as the mean of duplicate samples.

## RESULTS

Figure 1 reveals that in the presence of calcium the copolymer T130R2 causes significant net release of lysozyme and does not cause the release of cytoplasmic LDH after 30 minutes of incubation. This indicates that, as with

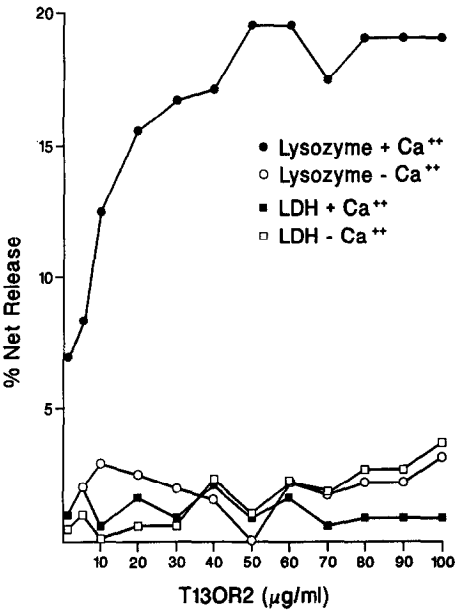
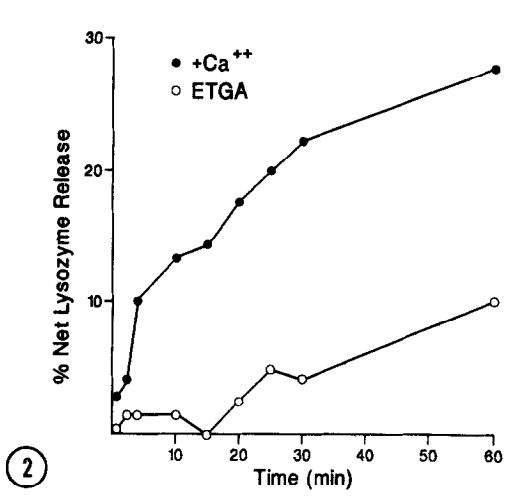



Fig. 1: Lysozyme release induced by T13OR2: Neutrophils were incubated for 30 minutes at 37° C with the indicated concentration of T13OR2 in the presence of 2mM $CaCl_2$ (closed Figures) or in buffer lacking calcium and containing 1 mM EGTA (open figures). The reaction is stopped and net lysozyme release determined as described in the methods. Shown are the percent of net lysozyme release (circles) and LDH release (squares) under these conditions. The results are the mean of two experiments performed in duplicate.

mouse mast cells, the polymer induces neutrophil granule exocytosis by a noncytotoxic mechanism. Figure 1 further demonstrates that the release is dose-dependent, with near maximal release occurring at a copolymer concentration of 10-30 ug/ml (1.25-3.75 uM). This closely parallels the effective concentration for mast cell histamine release (7), basophil histamine release (8), and ionophore activity (6). The release of neutrophil lysozyme by T13OR2 was dependent on the presence of calcium in the extracellular medium because marked inhibition was seen if the buffer contained 1 mM EGTA instead of calcium. Concentrations of T13OR2 up to 100 ug/ml were not cytotoxic as shown by LDH release of less than 1.5% in the presence of calcium.

We next evaluated the kinetics of T13OR2-induced lysozyme release. In Figure 2, neutrophils were incubated with T13OR2 (100 ug/ml) in the presence of 2 mM $Ca^{++}$ or in the absence of $Ca^{++}$ (+ 1 mM EGTA). Release was noted to occur within 2.5 minutes with the greatest portion of release occurring within the first 10 minutes. Substantial release continued for 30 minutes. After 30 minutes a slow component of enzyme release was observed through the 60 minute time point and was seen both in the presence and absence of extracellular calcium. Although the significance of this second phase is unclear, it is



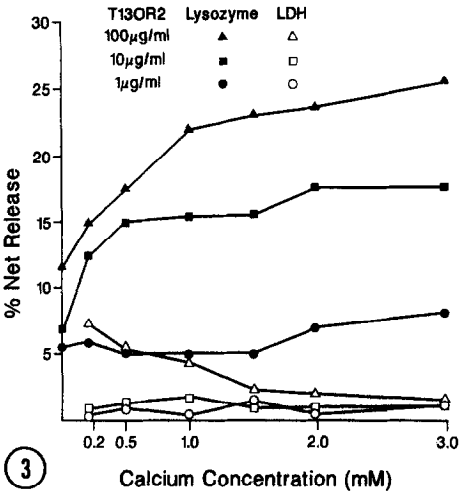




Fig. 2: Kinetics of lysozyme release: Neutrophils were incubated in the presence of 100 ug/ml of T13OR2 at 37° in the presence of 2 mM CaCl2 (closed circles) or in calcium free buffer (open circles), for the indicated time. The reaction is stopped by adding an equal volume of ice-cold calcium free buffer to each tube and placing the tubes on ice. Lysozyme is determined as described in the methods. The percent of net lysozyme release is shown and represents the mean of two separate experiments performed in duplicate.

Fig. 3: $Ca^{++}$ dependence of T13OR2-induced lysozyme release: Neutrophils were incubated in the presence of 1 (circles), 10 (squares), or 100 (triangles) ug/ml of T13OR2 in buffer containing the indicated concentration of calcium for 30 minutes. The reaction is stopped by placing the tubes on ice and the percentage of net release of lysozyme (closed symbols) or LDH (open symbols) determined. The results shown are a representative experiment performed in duplicate.

strikingly similar to the kinetics of basophil histamine release described previously (8) and could represent a delayed calcium-independent release of lysozyme or toxicity of the polymer.

Figure 3 is a representative experiment showing the results of varying the concentration of extracellular calcium on lysozyme release induced by three concentrations of T13OR2. It can be seen that over a thirty minute incubation the release of lysozyme showed a dose-dependent increase with increasing calcium ion concentration in the media. This is most apparent at the higher concentration of copolymer where enzyme release more than doubled at an optimal calcium concentration, 1 mM. This concentration is similar to that which is optimal for the activity of numerous other calcium-dependent stimuli, further indicating that the copolymer activates human neutrophils in a calcium-dependent manner.

We next determined whether T13OR2 would induce the neutrophil oxidative burst. At concentrations from 1 to 100 ug/ml T13OR2 did not induce neutrophils to reduce ferricytochrome C during a thirty minute incubation indicating a lack of superoxide production (data not shown).

## DISCUSSION

Available evidence indicates that the inflammatory activities of the group of synthetic polymers represented by T130R2 occur, at least in part, by the activation of mast cells and basophils. The present study demonstrates that T130R2 induces in a dose-, time-, and calcium-dependent manner the release of lysosomal enzymes from human neutrophils. Furthermore, studies in mice reveal that subcutaneous injection of T130R2 results in marked local inflammation, local mast cell degranulation, and a massive influx of neutrophils (manuscript in progress). In mice the injection of an oil-in-water emulsion containing 1.25 mg of copolymer results in peak footpad thickness over twice that of the normal foot (12). These data taken together indicate that neutrophils contribute substantially to the inflammatory reaction induced by the group of copolymers represented by T130R2.

Because the activation of cells by ionophores bypasses normal cell surface receptors, these agents typically express their activities over a wide array of cell types. Therefore the finding that T130R2 induces the release of neutrophil granule contents in addition to the previously noted histamine from mast cells is not incompatible with its action as an ionophore. In addition, the minimal effective concentration for neutrophil degranulation and other biologic activities thus far investigated are similar.

Previous studies have shown that T130R2 induces a rapid exchange of intracellular $K^+$ for extracellular $Na^+$ in human red cells with no change in the permeability of the cells to $Ca^{++}$ (6). Optimal release of histamine from murine mast cells, however, required the presence of extracellular $Na^+$ and $Ca^{++}$. These observations were interpreted to mean that the primary stimulus for cell activation with this agent is the influx of sodium ions with the requirement for extracellular $Ca^{++}$ hypothesized to depend on the activity of a membrane-associated Na-Ca exchanger (7). Evidence supporting the importance of this exchange process in neutrophil activation has been obtained in another laboratory (2).

The importance of depolarization and its relationship to other changes observed during neutrophil activation are incompletely understood. Depolarization induced by incubation of neutrophils in high potassium-containing buffer is sufficient to induce significant $Ca^{++}$ influx (13). Similarly, the divalent-selective ionophore A23187 produces a $Ca^{++}$- and $Na^+$-dependent membrane depolarization in several cells types which has been hypothesized to result from a $Ca^{++}$-dependent activation of sodium channels which cause the depolarization observed (4, 14-16). Optimal activation of neutrophils by A23187 requires the presence of extracellular $Na^+$(5), and removal of extracellular $K^+$ inhibits the release of lysozyme by A23187 (17),

indicative that the activation process is not simply a result of the ionophore-mediated influx of $Ca^{++}$.

The carboxylic ionophore monensin mediates primarily the exchanges of $H^+$ and $Na^+$ across cell membranes and is similar to T130R2 in that it induces a large increase in the intracellular $Na^+$ concentration. Monensin differs from T130R2 in that it does not dissipate transmembrane voltage gradients. Since the effects of monensin and T130R2 appear to differ primarily in their effects on the transmembrane potential (9), it is interesting that monensin does not cause significant activation of neutrophils, further supporting the importance of depolarization in some aspects of neutrophil activation.

We suggest that the degranulating activity of T130R2 and related compounds may depend on a depolarizing influx of $Na^+$ ions which stimulate $Ca^{++}$ entry via a membrane-associated Na-Ca exchanger. Activation of this ion exchange system in human neutrophils has recently been demonstrated during cell activation by f-met-leu-phe (2). Direct evidence that copolymer T130R2 causes membrane depolarization and an increase in the free intracellular calcium ion concentration will require further experiments.

## ACKNOWLEDGMENT

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