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Fluoride Stimulation of Canine Neutrophils: The Role of Calcium Binding[1] (41439)

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Abstract. Neutrophils are known to undergo a burst of metabolic activity in response to particulate and soluble stimuli. Most of these stimuli depend upon calcium in the extracellular medium to exert their maximal effects. We have shown that 24 m*M* sodium fluoride (NaF) stimulates the metabolic burst in canine neutrophils as measured by chemiluminescence and CO_2 formation from the carbon-1 position of glucose. Because these phenomena are calcium dependent, we determined the uptake of calcium by neutrophils activated with NaF. Neutrophils suspended in buffer were incubated with $^{45}CaCl_2$ and exposed to NaF. This caused an increase in cell-associated calcium from $1.89 \pm 0.43 \times 10^{-10}$ mol/10^7 cells to $1.06 \pm 0.15 \times 10^{-8}$mol/$10^7$ cells in 2 min. In comparison, the calcium ionophore A23187 (10^{-6} *M*) caused only a modest increase in cell calcium, from $1.89 \pm 0.43 \times 10^{-10}$ mol/10^7 cells to $4.89 \pm 0.85 \times 10^{-10}$ mol/10^7 cells. When treated with EGTA (3 m*M*), NaF-stimulated neutrophils rapidly lost 90% of their cell-associated calcium. Verapamil (3×10^{-4} *M*), a calcium channel blocker, inhibited NaF stimulation of neutrophil metabolism but did not decrease the magnitude or the rate of the calcium association. These studies indicate that the mechanism of NaF stimulation of neutrophils involves massive calcium binding to the external membrane and a subsequent movement of some of this membrane calcium into the cell.

During phagocytosis neutrophils undergo characteristic changes in metabolic activity including increased oxygen consumption, production of superoxide anion and hydrogen peroxide, and activation of the hexose monophosphate shunt. These metabolic changes have been reviewed by Babior (1). A variety of substances such as digitonin (2), saponin (3), fatty acids (4), and streptolysin-O (5) also induce neutrophil metabolic activity; presumably by reacting with the plasma membrane. Most of these stimuli appear to depend on extracellular calcium ions to achieve the maximum metabolic response.

The ability of sodium fluoride (NaF) to act as a metabolic stimulant of neutrophils was first recognized by Sbarra and Karnovsky (6). This stimulant is unusual in that the metabolic response can be rapidly terminated by removing extracellular fluoride (7). Stimulation by fluoride requires the presence of extracellular calcium but no other ions (7). Another unusual feature is the slowness of the metabolic response. Most neutrophil metabolic stimulants have a peak response within 5 min, while the metabolic activity in fluoride-treated cells generally has not begun by this time (8). Because of these characteristics, calcium uptake in relation to the metabolic response was examined in neutrophils activated with NaF. These observations were compared with another neutrophil stimulant, the calcium ionophore, A23187 (9).

Materials and Methods. *Isolation of neutrophils.* Blood was obtained from healthy mongrel dogs free of intestinal parasites. A 5% disodium ethylenediaminetetraacetate (EDTA) solution was used as an anticoagulant (0.1 ml EDTA/4 ml whole blood). After dilution with an equal volume of 0.9% saline, the blood was layered on a discontinuous Ficoll–Hypaque gradient using the method of Böyum (10). Following centrifugation the pellet containing red blood cells

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(RBC) and neutrophils was resuspended in saline. To every 10 ml of the resuspended pellet 3 ml of 5% dextran (average molecular weight = 500,000) was added, and the RBCs were allowed to settle out at 4° for 45 min. The neutrophil-rich supernatant solution was centrifuged for 10 min at 66*g*. The pellet was resuspended in ice-cold ammonium chloride buffer (NH_4Cl 155 m*M*; $KHCO_3$ 10 m*M*; EDTA 0.1 m*M*) for 10 min to lyse any contaminating RBCs. The cells were washed twice in calcium–magnesium-free Hank's balanced salt solution (HBSS), and resuspended in HBSS containing 0.9 m*M* magnesium ions and the desired calcium chloride concentration, usually 0.25 m*M*. The final cell preparation contained >95% polymorphonuclear leukocytes.

Assessment of neutrophil viability. Cell viability as assessed by trypan blue dye exclusion was routinely checked after each neutrophil isolation and after each calcium uptake experiment. Trypan blue uptake was determined by mixing equal volumes of trypan blue (0.1%) and neutrophils (1×10^6/ml), and counting cells containing trypan blue at 3 min. In all experiments 95% or more of the cells excluded the dye.

Measurement of light scattering. Because of the importance of avoiding calcium-containing precipitates during the calcium uptake studies, light scattering measurements were obtained. An Aminco Bowman spectrophotofluorometer (Model J4-8202 G) was utilized to study 90° light scattering. The exciting and reflected wavelengths were set at the same values, either 450, 500, or 550 nm. HBSS containing NaF (24 m*M*) and varying concentrations of $CaCl_2$ (0.05 to 0.50 m*M*) was examined for light scattering properties after equilibrating the solutions for 30 min at 37°. Significant scattering, taken to indicate CaF_2 precipitate formation, did not occur so long as the $CaCl_2$ concentration was at or below 0.45 m*M*.

Measurement of calcium uptake. Calcium uptake was measured by a modification of the technique described by Owen *et al.* (11). After a 30-min equilibration period at 37°, the neutrophil suspension (0.75 to 1.25×10^7 cells/ml) was supplemented with 0.8–1.2 μCi of $^{45}CaCl_2$ (0.6–0.8 mCi/μmol, Amersham, Arlington Heights, Ill.). In some experiments ^{125}I-bovine serum albumin (5–10 μCi/ml, Amersham) was added as an extracellular marker. In these experiments unlabeled bovine serum albumin (1%) prevented binding of the labeled albumin to the cells.

At predetermined time points following the addition of calcium-45, 1-ml aliquots of the suspension were withdrawn in quadruplicate and layered over silicone oil. The neutrophils were pelleted by centrifugation (Fisher Model 59) at 7000*g* for 1.5 min (12).

Additional samples were removed at varying intervals after the introduction of A23187 (10^{-6} *M*) or NaF (24 m*M*). Neutrophil pellets and 100-μl aliquots of cell-free supernatants were solubilized in 0.5 ml Soluene 350 (Packard Instrument Co., Downers Grove, Ill.) and counted for 500 sec in a Packard Auto Gamma spectrometer to measure ^{125}I activity. ACS (Amersham) scintillation fluid was added, and samples were counted in a liquid scintillation spectrometer (Packard Model 3255) to measure calcium-45 activity. A window setting of 300–1000 with a gain of 14% permitted the selective counting of calcium-45 in the presence of iodine-125.

The extracellular space in the pellet of cells did not significantly change over the course of a given experiment for resting cells, exhibiting an average space of 0.32 ± 0.01 μl/10^7 cells. However, when stimulated, the average extracellular space increased to 0.45 ± 0.05 μl/10^7 cells. After appropriate correction for trapped buffer, the volume of the neutrophil ^{45}Ca "space" was calculated using a formula from Feinberg *et al.* (13). The neutrophil ^{45}Ca "space" multiplied by the calcium concentration of the medium determined the cell-associated calcium value (moles of calcium per 10^7 neutrophils).

Chemiluminescence. Chemiluminescence was measured in a specially designed chemiluminescence spectrometer which maintained the temperature of the reaction at 37° and provided continuous mixing of the cell preparations (14). The neutrophil

concentration was 1×10^6 cells/ml, and the cells were suspended in HBSS containing 0.25 m*M* or no $CaCl_2$. Luminol (10^{-8} *M*) was used to augment the chemiluminescent response. Light emission was recorded as counts per unit time.

Glucose metabolism. Glucose metabolism through the hexose monophosphate shunt was evaluated using [1-^{14}C]glucose (61.1 mCi/mmol; Amersham) as described in a previous study (15). The results were expressed as counts per minute (cpm) of ^{14}C activity per 5×10^6 cells. Baseline metabolic activity of unstimulated neutrophils (generally about 12–15% of the stimulated value) was subtracted from the results of stimulated cells.

Reagents. All chemicals were of the best quality commercially available. Hank's balanced salt solution was obtained from Gibco, Grand Island, New York. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) was purchased from Eastman Kodak Company, Rochester, New York. Ethylene glycol bis-(β-aminoethylether)-*N,N'*-tetraacetic acid (EGTA), and sodium fluoride were obtained from the Sigma Chemical Company, St. Louis, Missouri. A23187 and verapamil were provided by Robert L. Hamill, Ph.D. Lilly Research Laboratories, Indianapolis, Indiana, and Edward B. Kirsten, Ph.D., Knoll Pharmaceutical Company, Whippany, New York, respectively. A23187 was dissolved in dimethyl sulfoxide at a concentration of 10^{-3} *M* and stored at −30°. All other solutions were freshly prepared for each experiment.

Statistical analysis. The group Student's *t* test was used to analyze replicate observations in this study. All mean values are followed by ± standard error of the mean.

Results. *Stimulatory effects of A23187 and NaF.* Unstimulated neutrophils slowly took up calcium-45 and approached equilibrium at 40 min. The average value of cell-associated calcium at 40 min was $1.89 \pm 0.43 \times 10^{-10}$ mole of $Ca^{2+}/10^7$ cells ($n = 11$). Typical equilibration curves can be seen in Fig. 1 and 2. In all experiments A23187 and NaF were added 40 min after the addition of calcium-45.

The calcium ionophore A23187 (10^{-6} *M*), promoted a rapid increase in neutrophil calcium content. Within 2 min after stimulation, calcium levels rose from the resting level to $4.89 \pm 0.85 \times 10^{-10}$ mole/10^7 cells ($n = 3$). One experiment is shown in Fig. 1B.

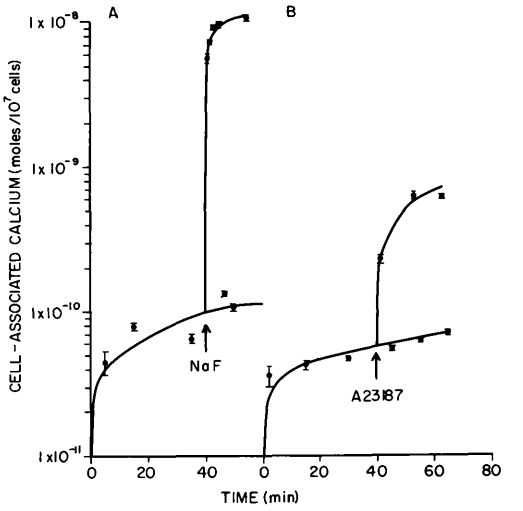

FIG. 1. Calcium uptake by NaF and A23187-stimulated neutrophils. Forty minutes after the addition of $^{45}CaCl_2$, 24 m*M* NaF(graph A) and 10^{-6} *M* A23187 (graph B) were added. The cells were maintained in Hank's balanced salt solution with 0.25 mM $CaCl_2$ at 37° with mixing throughout the experiment. Each data point represents the mean of four determinations ± the standard error.

The peak chemiluminescent response of neutrophils to A23187 (10^{-6} *M*) in the presence of 0.25 m*M* Ca^{2+} was 82,000 cpm. In the absence of extracellular calcium ions the amount of emitted light was reduced to a peak response of 26,000 cpm. Maximal chemiluminescence occurred within 5 min following the addition of A23187.

NaF (24 m*M*) also caused calcium uptake by the neutrophils (Fig. 1A); however, this uptake occurred at a much faster rate and reached much higher levels than those seen with A23187. Within 2 min after NaF stimulation the average cell-associated calcium levels had reached $1.06 \pm 0.15 \times 10^{-8}$ mole/10^7 cells ($n = 9$), 56 times the calcium uptake of the resting cells and 22 times the calcium uptake of neutrophils 2 min after A23187 stimulation. The possibility that NaF was causing a marked increase in

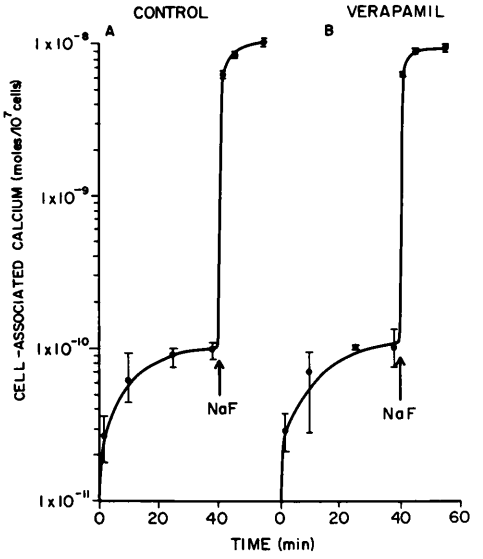

FIG. 2. The effect of verapamil (3×10^{-4} *M*) on the calcium uptake of neutrophils stimulated by 24 m*M* NaF added 40 min after $^{45}CaCl_2$. In graph A only NaF was used while in graph B verapamil was added to the cell suspension 5 min prior to the addition of the $^{45}CaCl_2$. The cells were maintained in Hank's balanced salt solution with 0.25 m*M* $CaCl_2$ at 37° with mixing throughout the experiment. The data points plotted prior to NaF addition are the mean of two determinations ± the range. Data points after the addition of NaF represents the mean of four determinations ± the standard error.

membrane binding of calcium was suggested by this large and rapid calcium uptake. The NaF-induced chemiluminescence of neutrophils showed a calcium ion dependence which was similar to A23187. The peak response to NaF was 610,000 cpm in the presence of 0.25 m*M* Ca^{2+}, but only 99,000 cpm in the absence of extracellular calcium. The NaF-induced chemiluminescence response began 16 min after the addition of NaF, and reached a peak of activity at 52 min.

In addition to chemiluminescence, hexose monophosphate shunt activity was determined in NaF-stimulated cells. Neutrophils suspended in HBSS with 0.25 m*M* $CaCl_2$ and treated with 24 m*M* NaF had large increases in shunt activity as measured by carbon-14 liberated from the C-1 position of glucose/5 × 10^6 cells. NaF-stimulated cells produced 2424 ± 587 cpm/5 × 10^6 cells during a 40-min incubation period. Thus we have determined that NaF stimulation resulted in not only a large calcium uptake but have confirmed the activation of metabolism, as measured by both chemiluminescence and hexose monophosphate shunt activity.

Effects of verapamil on NaF-stimulated neutrophils. If the metabolic response to NaF is the result of an influx of calcium ions into the cell, it would be expected that a calcium channel blocker such as verapamil would block the calcium influx and related metabolic stimulation. As shown in Fig. 2, verapamil (3×10^{-4} *M*) had no effect on NaF-stimulated calcium uptake.

In chemiluminescence studies, however, verapamil, when added 5 min after NaF, completely blocked light production (Fig. 3). If the verapamil was added during the chemiluminescent response (12 min later), there was a prompt termination of chemiluminescence (Fig. 3). Verapamil added prior to NaF also prevented the chemiluminescent response (data not shown).

The direct interaction of verapamil with chemical intermediates of activated neutrophils was evaluated as a possible cause of the suppressed chemiluminescence. To accomplish this, the effect of verapamil on two cell-free chemiluminescent chemical reactions was examined (16). The interaction of H_2O_2 and NaOCl is known to produce a singlet oxygen intermediate, and $FeSO_4$ and H_2O_2 has a hydroxyl radical intermediate (16). The chemiluminescence of these systems augmented with luminol were tested in the presence and absence of verapamil. H_2O_2 (0.1 *M*) was added to NaOCl(0.2 *M*), and luminol (10^{-8} *M*) with or without verapamil (3×10^{-4} *M*). In the absence of verapamil there were 88,877 ± 3980 counts in the first 4 sec of the experiment. When verapamil was present 89,986 ± 5566 counts in 4 sec were detected. Thus it appears unlikely that verapamil quenched the intermediates of the H_2O_2–NaOCl reaction. In the case of $FeSO_4$–H_2O_2 chemiluminescence, verapamil in the presence of luminol generated 30,992 ± 1456 counts in the first 4 sec of the reaction.

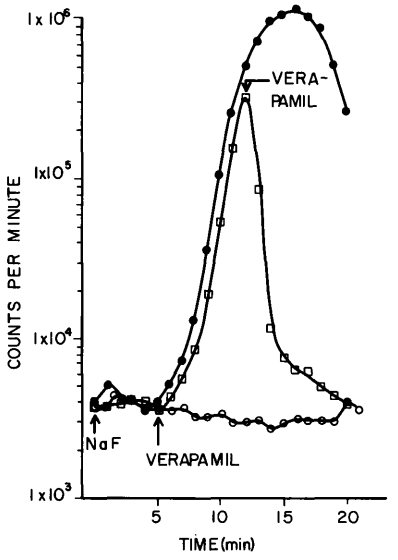

FIG. 3. The effect of verapamil on neutrophil chemiluminescence stimulated by NaF. Verapamil (3×10^{-4} *M*) was added either 5 (○) or 12 (□) min after NaF (24 m*M*). (●) NaF only. All cells were kept at 37° with continuous mixing.

$FeSO_4$ plus H_2O_2 with luminol but no verapamil gave 6551 ± 195 counts in 4 sec ($P < 0.001$). From this it appears that verapamil may augment the chemiluminescence of some oxygen intermediates but there is no apparent quenching.

The possibility that verapamil was blocking the movement of calcium from the cell surface into the cytoplasm and as a consequence suppressing metabolic activity received additional support from measurements of C-1 glucose metabolism. This metabolic response was reduced from 2424 ± 587 to 324 ± 174 cpm/5 × 10^6 cells ($P < 0.01$) when verapamil (3×10^{-4} *M*) was added 5 min after NaF stimulation was initiated. A similar reduction in activity occurred at a verapamil concentration of 1×10^{-4} *M* but not at 1×10^{-5} *M* (data not shown).

Effects of EGTA on NaF-stimulated neutrophils. The fact that verapamil could block the NaF-induced metabolic response, as measured by chemiluminescence and C-1 glucose metabolism, but not calcium uptake, suggested that the principal NaF effect was to induce binding to the exterior of the neutrophil membrane. If this is so, calcium chelation would be expected to rapidly reverse the observed calcium uptake. EGTA (3 m*M*), a calcium chelator, when added 2 min after fluoride stimulation rapidly removed 90% of the cell-associated calcium within 3 min after adding the EGTA (Fig. 4). In another experiment when calcium uptake was determined immediately, 30 sec, and 1 min after the addition of EGTA which had been added 1 min after NaF, the levels of uptake were reduced by 90% or more when compared with cells treated only with NaF.

EGTA (3 m*M*) was less efficient in blocking NaF-induced chemiluminescence than verapamil. There was relatively little decrease in light production in the presence of EGTA when the neutrophils were suspended in HBSS containing 0.25 m*M* $CaCl_2$. When the calcium concentration was reduced to 0.025 m*M* and the EGTA concentration remained the same, light production by neutrophils was only 28% of that produced by NaF-stimulated cells in the absence of EGTA.

In a study to determine the effect of calcium chelation on C-1 glucose metabolic activation by NaF, neutrophils were suspended in HBSS with 0.025 m*M* $CaCl_2$. When these cells were treated only with NaF (24 m*M*) 344 ± 63 cpm/5 × 10^6 cells were produced in a 45-min incubation period. When EGTA (3 m*M*) was added 5 min after NaF (24 m*M*) only 108 ± 7 cpm/5 × 10^6 cells were produced in a similar incubation period ($P < 0.01$).

Discussion. Our results confirm previous observations (7) that calcium is essential for maximal fluoride stimulation of the neutrophil metabolic burst. We have found that calcium is taken up by NaF-stimulated cells in amounts significantly greater than that seen with other soluble stimuli. Although large amounts of calcium were induced to bind to neutrophils by NaF, we conclude that most of the bound calcium remained external to the cell. This is based on our findings using EGTA, and verapamil.

EGTA is a large, polar molecule that does not readily cross cell membranes. The

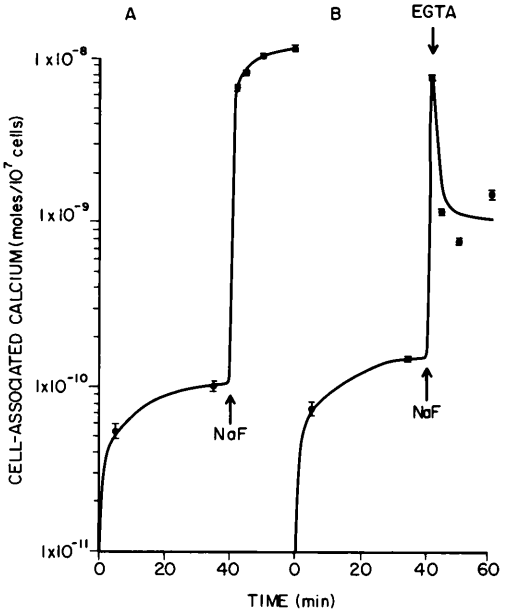

FIG. 4. The effect of EGTA on NaF-induced calcium uptake in neutrophils. NaF (24 m*M*) was added to neutrophils suspended in Hank's balanced salt solution with 0.25 m*M* $CaCl_2$ and equilibrated with $^{45}CaCl_2$ for 40 min. EGTA (3 m*M*) was added (graph B) 2 min after NaF treatment. All data points represent the mean of four determinations ± the standard error.

rapid decrease in cell-associated calcium observed after EGTA treatment must, therefore, reflect the chelation of easily accessible, membrane-bound calcium. The inability of EGTA to block the metabolic response until the extracellular calcium level was reduced, was probably due to incomplete calcium chelation.

Verapamil, a membrane channel blocker (17), has been shown to inhibit calcium uptake by neutrophils exposed to formyl-methionyl-leucyl-phenylalanine (18) as well as neutrophil activation. Verapamil, as shown in the present experiments, effectively inhibited NaF-induced chemiluminescence and activation of C-1 glucose metabolism, but it had no inhibitory effect on NaF-induced calcium uptake. The apparent paradox of the failure of verapamil to influence calcium uptake while successfully blocking the metabolic response seems to be due to a biphasic calcium movement. The first phase, which involves binding of calcium to the external surface of the plasma membrane, is not influenced by verapamil, while the second phase, the internalization of a portion of the plasma membrane calcium, is blocked by verapamil. Available evidence indicates that verapamil prevents calcium transport across cell membranes by blocking "slow" calcium channels (19). The exact mechanism has not been established but recent work with verapamil analogs suggests that its site of action is on the cytoplasmic side of the plasma membrane (20). Thus, verapamil would not be expected to interfere with calcium binding to the plasma membrane but rather the cellular processes that mediate the transport of calcium across the plasma membrane and into the cytosol.

Neutrophil chemiluminescence induced by fluoride is delayed when compared with other soluble stimuli. A23187-stimulated chemiluminescence begins shortly after addition of the stimulus and reaches a peak in 3 to 5 min. Other soluble and particulate stimuli behave in a similar fashion. When fluoride is the stimulus a lag period of at least 5 to 7 min occurs and the peak of light production is not reached for about 15 min or longer. A comparison of the calcium uptake and chemiluminescence of NaF and A23187 provides some interesting contrasts. When NaF is the stimulus there is significant lag seen between calcium uptake (the rapid event) and chemiluminescence (the delayed event). This differs from A23187 stimulation where calcium uptake and chemiluminescence occur at virtually the same time. It appears, therefore, that when NaF is the stimulus a second reaction must ensue subsequent to the rapid phase of calcium uptake and prior to activation.

The results described suggest the following mechanism for the stimulation of the metabolic burst by fluoride. Fluoride promotes a massive, rapid, binding of calcium to the neutrophil plasma membrane. Since neutrophil membranes are already known to bind calcium (21) fluoride may be exposing additional calcium binding sites. Some of this calcium slowly moves across the membrane into the cell via verapamil-

sensitive channels. When the cytosolic calcium concentration reaches an appropriate level, it activates a calcium sensitive enzyme or enzyme system that in turn initiates the metabolic burst. This would account for the lag between calcium binding and the onset of the metabolic response, and the action of verapamil to inhibit NaF activation despite the fact that calcium binding was not prevented. However, there may be other explanations for fluoride stimulation of metabolism. Selvaraj and Sbarra (22) have postulated that fluoride may move into the cell and form a magnesium–fluoro–phosphate complex which could lead to metabolic activation. This concept awaits the measurement of intracellular fluoride levels before it can be adequately evaluated.

Curnutte *et al.* (7) found that superoxide anion production by human neutrophils treated with fluoride was reversible. Stimulated neutrophils could be returned to a resting state simply by washing the cells. The addition of fresh fluoride reestablishes renewed superoxide anion production. This reversibility is compatible with the model described here. Washing the fluoride-treated cells is likely to remove the calcium bound to the membrane. Without the additional membrane bound calcium, the calcium level in the cytosol drops below that required to maintain the metabolic burst. The addition of fresh fluoride reestablishes the higher level of membrane-bound calcium and the subsequent reactivation of the metabolic burst.

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