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INCREASES IN CYTOSOLIC CALCIUM ION LEVELS IN HUMAN NATURAL KILLER CELLS IN RESPONSE TO BUTYLTIN EXPOSURE

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Abstract

This study investigated whether exposures to butyltins (BTs), tributylin (TBT) and dibutyltin (DBT) were able to alter cytosolic calcium levels in human natural killer (NK) cells. Additionally, the effects of cytosolic calcium ion increases on the activation state of mitogen activated protein kinases (MAPKs) in NK cells were also investigated. NK cells are an intital immune defense against the development of tumors or viral infections. TBT and DBT are widespread environmental contaminants, due to their various industrial applications. Both TBT and DBT have been shown to decrease the ability of NK cells to lyse tumor cells (lytic function). TBT has also been shown to activate MAPKs in NK cells. The results of this study indicated that TBT increased cytosolic calcium levels by as much as 100% after a 60 min exposure to 500 nM TBT while DBT increased cytosolic calcium levels to a much smaller extent (and required higher concentrations). The results also indicated that increases in cytosolic calcium could activate MAPKs but only for a short period of time (5 min), while previous studies showed that activation of MAPKs by TBT last for at least 6 hours. Thus, it appears that TBT stimulated increases in cytosolic calcium may contribute to, but are not fully responsible for, TBT-induced activation of MAPKs.

INTRODUCTION

Natural killer (NK) cells are a subset of lymphocytes responsible for innate, or non-adaptive, immunity. They are found primarily in the bone marrow, spleen, liver and peripheral blood, contributing 5 – 20% of total lymphocytes in the peripheral blood system (Moretta et al., 2002). NK cells are defined by the absence of the T cell receptor/CD3 complex and by the presence of the CD56 and/or CD16 on the cell surface (Lotzova, 1993). NK cells play a central role in immune defense against viral infection and formation of primary tumors (Lotzova, 1993; Vivier et al., 2004). NK cells are responsible for limiting the spread of blood-borne metastases as well as limiting the development of primary tumors (Lotzova, 1993). NK cells also play a central role in immune defense against viral infection as evidenced by increased incidences of viral infection seen in individuals where the NK subset of lymphocytes is completely absent (Fleisher et al., 1982; Biron et al., 1989). These cells are the front line of immune response against tumor and virally infected cells due to their ability to lyse appropriate target cells without prior sensitization. Interference with NK-cell function by any compound could increase the risk of viral infection and tumor formation.

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Butyltins (BTS) are man-made chemicals used in a variety of industrial, aquacultural, and consumer applications that cause significant contamination of the global environment (Laughlin and Linden, 1985; Tanabe et al., 1998; Senthil Kumar et al. 1999a). Tributyltin (TBT) was produced in large quantities for wood preservation, marine antifouling paints, disinfection of circulating industrial cooling water, and slime control in paper mills (Roper, 1992). The use of TBT in antifouling paint applications is responsible for release of these compounds into the aquatic environment (Hall and Pinkney; 1985; Stallard et al., 1987; Clark et al., 1988). Detectable levels of TBT have been found in fish and birds (Alzieu et al., 1989; Kannan et al, 1995a,b,c; Kannan and Falandyz, 1997; Senthil Kumar et al. 1998, 1999b). It has also been found in plastic products (baking parchments made from siliconized paper) that come in contact with food and was found to transfer into cookies placed on TBT-containing baking parchments (Takahashi et al., 1999). There is significant exposure of humans to TBT as evidenced by its detection in human blood (Whalen et al., 1999; Kannan et al., 1999). Tumors have been shown to be produced from tributyltin oxide exposure in a mammalian model (Wester et al., 1990). BT exposure has also been shown to disrupt the immune system of marine mammals (Nakata et al., 2002). DBT is used as a stabilizer in the production of polyvinyl chloride (PVC) plastic products (Roper, 1992) and has been found in some plastic food containers (Nakashima et al., 1990; Yamada et al., 1993). It has also been used as a deworming agent in poultry and some poultry products have been shown to contain measurable levels of DBT (Epstein et al., 1991). Drinking water has also been reported to contain DBT due to leaching from PVC pipes (Sadiki et al., 1996). Human exposure to DBT could come from poultry products (Epstein et al., 1991), consuming beverages stored in PVC pipes during manufacture (Forsyth et al., 1992a,b), and drinking water (Sadiki et al., 1996). DBT has also been found in human blood from donors with no specific route of exposure other than what is found in the environment (Kannan et al., 1999; Whalen et al., 1999). It has also been shown to cause atrophy of the thymus and pancreatitis in exposed rats (Pieters et al., 1994; Merkord et al., 1997). Exposure of animals to DBT may debilitate their immune function, making them more vulnerable to infectious diseases (Kannan et al. 1997, 1998). The presence of TBT and DBT in human blood (Whalen et al., 1999; Kannan et al., 1999) raises serious concerns since we have shown in previous studies in our laboratory that BT have deleterious effects on the ability of NK cells to perform their tumor lysing function (Whalen et al., 1999; Dudimah et al., 2007a,b).

NK cells destroy target cells by releasing granules containing cytotoxic proteins, perforin and granzyme B, onto target cells (Podack, 1992; Talanian et al., 1997). When an NK cells binds to a target cell a series of enzymatic activities are stimulated in the NK cell these appear to include activation of protein kinase C (PKC) (Chuang et al., 2003; Steele and Brahmi, 1988; Procopio et al. 1989) and activation of mitogen activated protein kinases (MAPK) (Trotta et al, 1998; Trotta et al., 2000). PKC can be activated by increases in cytosolic calcium ion (Ca^{2+}) as well as by diacylglycerol (DAG) or a combination of elevated Ca^{2+} and DAG (Steinberg, 2008). Thus, there is a potential role for increased cytosolic calcium levels in the activation of PKC which could then lead to a series of activations resulting in activation of MAPKs (Pearson et al., 2001). This MAPK activation is able to stimulate granule release (Trotta et al., 1998). Our previous studies have shown that one of the consequences of TBT exposures was activation of MAPKs in human NK cells (Aluoch and Whalen, 2005; Aluoch et al., 2006; Aluoch et al., 2007). This TBT-induced activation of MAPKs could leave the NK cell unable to respond to target cells. The mechanism by which TBT causes the activation of MAPKs has not been elucidated. TBT-induced elevation of cytosolic [Ca^{2+}] in NK cells could potentially lead to activation of MAPKs. TBT has been shown to alter cytosolic Ca^{2} in other cell types (Chikahisa and Oyama, 1992; Chow et al., 1992; Stridh et al., 1999; Kawanishi et al., 2001).

The aim of this study is to determine the effects of brief exposures to the butyltins, TBT and DBT, on the $[Ca^{2+}]_{cyt}$ in NK cells. This will elucidate whether exposures to BTs induce increased $[Ca^{2+}]_{cyt}$ in NK cells as has been seen in thymocytes (Chow et al., 1992). An additional aim of this study is to determine whether an increase in cytosolic Ca^{2+}, stimulated by the calcium ionophore A23187, is able to activate MAPK activation in NK cells. If MAPK are activated by the ionophore, it will be determined if the extent of activation is similar to that induced by TBT exposures.

MATERIALS AND METHODS

Preparation of human NK cells

Peripheral blood from healthy adult (male and female) volunteer donors was used for this study (Red Cross, Portland, OR). Buffy coats were shipped overnight at ambient temperature and processed immediately upon receipt. Highly purified NK cells were obtained using a rosetting procedure. Buffy coats were mixed with 0.8 ml of RosetteSep human NK cell enrichment antibody cocktail (StemCell Technologies, Vancouver, BC, Canada) per 45 ml of buffy coat. The mixture was incubated for 25 min at room temperature (25° C) with periodic mixing (approximately every 20 min). Following the incubation, 5 ml of the mixture was layered onto 4 ml of Ficoll-Hypaque (1.077 g/ml) (Sigma-Aldrich, St. Louis, MO) and centrifuged at 1200 g for 30 min. The cell layer was collected and washed twice with phosphate buffered saline (PBS) and stored in complete media (RPMI-1640 supplemented with 10% heat-inactivated BCS, 2 m*M L*-glutamine and 50 U penicillin G with 50 μg streptomycin/ml) at 1 million cells/ml. The resulting cell preparation was >95% CD16+, 0% CD3+ by fluorescence microscopy and flow cytotometry (Whalen et al., 2002; Bariagaber and Whalen, 2003).

Chemical preparation

TBT and DBT were purchased from Aldrich Chemical Co. (Milwaukee, WI). DBT was dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich) to give a stock solution. TBT is a liquid at room temperature and was dispersed initially in double de-ionized water (ddH_2O) to give a 1 mM solution. Desired concentrations of each of the compounds were prepared by dilution of the stock into the appropriate medium.

Flow Cytometry

NK cells were incubated with 5μM Fluo-3, AM (Molecular Probes, Eugene, OR), for 1 h at a concentration of 2 million cells/mL. Following the incubation the cells were washed three times with either RPMI 1640 or Phosphate Buffered Saline (PBS, without Ca^{2+} plus 2 mM EDTA) and stored on crushed ice (Scotsman Ice Systems, Vernon Hills, IL). The samples were then exposed to varying concentrations of TBT (25 – 500 nM) or DBT (0.5- 10 μM) for varying lengths of time (3 – 120 min) and analyzed by Flow Cytometry. Samples were analyzed using the FACSCalibur flow cytometer from Becton Dickinson Immunocytometry Systems, Inc (BDIS), San Jose, CA. Instrument performance was standardized weekly using Calibrite beads (BDIS) and the same instrument settings were used for all acquisitions. The assays were sufficiently uniform to use the same FSC, SSC, and FL settings. The sensitivity of the instrument was constant. The acquisition and analysis software for flow cytometry data was CELLQuest Pro from BDIS running on an Apple computer.

Cell lysates for Western Blots

NK cells at a concentration of 6 million/mL will be incubated for 0-60 min with 5 μM A23187. Following the above treatments, the cells were centrifuged and the cell pellets were lysed using 133 μL of lysis buffer (Active motif, Carlsbad, CA) per 2 million cells. The cell lysates were stored frozen at −80 °C up to the point when they were run on SDS-PAGE. Control and treated

cells for a given experimental setup (described above) were from an individual donor (Aluoch and Whalen, 2005).

Western Blots

Cell lysates prepared as described were run on 10% SDS-PAGE and transferred to PVDF membranes. The PVDF membranes were immunoblotted with anti-phospho p44/42 and anti-p44/42 antibodies (Cell Technologies Inc., Beverly, MA). The density of antibodies was visualized using ECL chemiluminescent detection system and a Kodak Imaging System (Kodak, Rochester, NY). β-actin levels were determined for each condition to verify that equal amounts of protein were loaded. In addition, the density of each protein band was normalized to β-actin to correct for small differences in protein loading among the lanes (Aluoch and Whalen, 2005).

Statistical Analysis

Statistical analysis of the data was carried out utilizing ANOVA and Student's t test. Data were initially compared within a given experimental setup by ANOVA. A significant ANOVA was followed by pair wise analysis of control versus exposed data using Student's t test.

RESULTS

Effect of TBT exposures on Ca^{2+} levels in NK cells

NK cells loaded with Fluo3 AM were exposed to 500, 300, 200, 100, 50 and 25 nM TBT for periods of 3, 15, 30, and 60 min. Figure 1A shows the average increases in intracellular Ca^{2+} as compared to control cells. The studies shown in Figure 1A were carried out in cell culture medium (RPMI 1640, Mediatech Inc., Herndon, VA) which contained 0.42 mM Ca^{2+}. Additional studies were carried out in a PBS solution which had no added Ca^{2+} and in which there was 2 mM EDTA. As seen in figure 1A, when NK cells were exposed to 500 nM TBT there was an average increase in cytosolic Ca^{2+} of 43.9±15.7% at 3 min and this increased to 98.1±41.5% by 30 min. NK cells exposed to 300 nM TBT showed an average increase in cytosolic Ca^{2+} of 37.8±8.9% at 3 min and 42.3±10.1 at 30 min. The figure is an average of 4 separate experiments using different donors. Figure 1B shows shifts in Fluo-3AM flourescence with a 30 min exposure of NK cells to 500 nM TBT and Figure 1C with a 300 nM exposure to TBT from a representative experiment. The greatest increase in Ca^{2+} occurred at either 30 min or 60 min, in any given experiment for the 500 and 300 nM concentrations. Exposures to 200 and 100 nM TBT for 3 min caused increases in NK-cell-cytosolic Ca^{2+} of 35.8±7.0% and 29.2±7.2 %, respectively. 30 min exposures to 200 and 100 nM TBT caused increases of 28.2 ±8.7% and 18.8±11.3%, respectively. Unlike 500 and 300 nM exposures the increases were normally greatest at the 3 minute time point when NK cells were exposed to 200 and 100 nM TBT. Figure 1D shows the increase in Fluo-3AM fluorescence when NK cells were exposed to 200 nM TBT for 3 min and Figure 1E shows the effects of a 3 min exposure to 100 nM TBT. NK cells exposed to 50 nM TBT showed small but significant increases in cytosolic Ca^{2+} of 14.4±5.9% at 3 min that did not maintain out to 30 min. There was no significant increase in cytosolic Ca^{2+} when NK cells were exposed to 25 nM TBT at any time point.

Figure 2 compares the increases in cytosolic Ca^{2+} when there was 0.42 mM Ca^{2+} (RPMI1640) versus no Ca^{2+} (PBS solution which had no added Ca^{2+} and in which there was 2 mM EDTA). Figure 2A shows that the increase in cytosolic Ca^{2+} seen with a 30 min exposure to 500 nM TBT was reduced by greater than 88% when there was no Ca^{2+} in the extracellular fluid. Increases in cytosolic Ca^{2+} stimulated by 30 min exposures to 300 nM (Figure 2B) and 200 nM TBT (Figure 2C) were both reduced by greater than 70%.

Effect of DBT exposures on Ca $^{2+}$ levels in NK cells

NK cells loaded with Fluo3 AM were exposed to 10, 5, 2.5, 1, and 0. 5 μM DBT for periods of 3, 15, 30, and 60 min. Figure 3A shows that there were small by significant increases in cytosolic Ca^{2+} when NK cells were exposed to 10, 5,and 2.5 μM DBT for 30 min. The increases were about 20% at 10 μM, 13% at 5 μM, and 8% at 2.5 μM DBT. There were also increases seen with exposures for 60 min to 10, 5 and 2.5 μM DBT (Figure 3A). There were no significant increases in cytosolic Ca^{2+} when exposures were for 3 or 15 min. These increases were measured when the cells were exposed to DBT in media containing 0.42 mM Ca^{2+} (RPMI 1640). Figure 3B shows the increase in Fluo-3AM fluoresecence with an exposure of NK cells to 10 μM DBT for 30 min from a representative experiment.

Effect of exposures to 5 μM A23187 on Ca $^{2+}$ levels in NK cells

NK cells loaded with Fluo3 AM were exposed to the calcium ionophore, A23187, (5 μM) for 3, 15, 30 and 60 min. Figure 4A shows the percent increase in cytosolic Ca^{2+} at each of these time points when the NK cells were suspended in calcium containing media (RPMI 1640). The increase was greatest at 3 min being about 250% greater than control cells. Figure 4B shows the increase in Fluo-3AM fluoresecence with an exposure of NK cells to 5 μM DBT for 3 min from a representative experiment.

Effect of exposures to 5 μM A23187 on mitogen activated protein kinase (MAPK) activation in NK cells

Figure 5 shows the effects of the increase in cytosolic Ca^{2+} caused by treatment with 5μM A23187 on the activation states of the MAPKs p44/42 and p38. NK cells were treated with A23187 for 0, 5, 10, 30 or 60 min. Figure 5A indicates that there was a statistically significant increase in p44/42 phosphorylation of about 2 fold 5 min after exposure to the ionophore ($p<0.025$). A 5 min exposure to 5μM A23187 also caused an significant increase in p38 phosphorylation, 2.6 fold ($p<0.025$) (figure 5A). Figure 5B is a representative experiment. There were no significant changes in JNK phosphorylation at any time point.

DISCUSSION

The aim of these studies was to determine if exposures to the butyltins, TBT and DBT were able to cause changes in the levels of cytosolic Ca^{2+}in human NK cells. Further, they examined whether increases in cytosolic Ca^{2+} in NK cells stimulated by a calcium ionoophore, A23187, could cause alterations of MAPK activation similar to those seen with TBT.

Previous studies have shown that TBT increased intracellular Ca^{2+} in thymocytes from rats (Chow et al., 1992) and mice (Chikahisa and Oyama; 1992) and in a mutated T cell (Stridh et al., 1999). However there have been no studies examining the effects of TBT on cytotsolic Ca^{2+} in normal human lymphocytes. Our earlier studies have indicated that exposures to TBT ranging from 500-25 nM are able to decrease the ability of human NK lymphocytes to carry out their crucial function of destroying tumor cells (Whalen et al., 1999; Whalen et al., 2002; Dudimah et al., 2007a). We have found that these same exposures were able to lead to activation of several MAPK within 5-10 minutes (Aluoch and Whalen, 2005; Aluoch et al., 2006; Aluoch et al., 2007). MAPK activation can lead to the release of cytotoxic proteins from NK cells onto tumor cells, which then cause tumor cell destruction (Trotta et al, 1998; Trotta et al., 2000). TBT-induced activation of MAPK could then leave the NK cell unable to respond to tumor cell contact. MAPK are activated by MAPK kinases (MAP2K), which are in turn activated by MAP2K kinases (MAP3K) (Vivier et al., 2004). MAP3K have the capacity to be activated by PKC (Pearson et al., 2001) which under some circumstances can be activated by an increase in cytosolic Ca^{2+}, as well as other mechanisms (Steinberg, 2008). Thus, it was important to examine whether TBT induces alteration in cytosolic Ca^{2+} in NK cells and to examine whether

increased Ca^{2+} could lead to activations of MAPKs similar to those seen with TBT exposures (Aluoch and Whalen 2005; Aluoch et al., 2006; Aluoch et al., 2007).

The current study showed that TBT exposures of 500 nM caused increases in cytosolic Ca^{2+} of approximately 100% after 60 min. Exposures to 300 and 200 nM TBT caused increases of about 40% after 60 min and exposure to 100 nM TBT caused an increase of about 20% after 60 min. It appeared as though the increases in cytosolic Ca^{2+} were mainly due to an influx of extracellular Ca^{2+} rather than release from intracellular sources, since the increase in cytosolic Ca^{2+} seen at 60 min with 500 nM TBT in media where Ca^{2+} had been minimized by addition of EDTA was less than 10% of that seen in Ca^{2+} -containing medium. The increases in cytosolic Ca^{2+} seen with 300 and 200 nM were also greatly diminished when there was no Ca^{2+} added to the extracellular media. There were no measureable increases at 100 and 50 nM TBT when the experiment was done in Ca^{2+} free media. Additionally, the data indicated that the TBT-induced increase in cytosolic Ca^{2+} began to decline after 60 min. DBT is also able to decrease the lytic function of human NK cells at concentrations ranging from 10 -0.5 μM (in the range of 20 times greater concentrations than are needed with TBT) (Whalen et al., 1999; Dudimah et al., 2007b). The effects of DBT exposures on cytosolic Ca^{2+} were also examined and it was found that both 10 and 5 μM DBT could cause significant but quite small increases after 30 and 60 min. The increases were about 20% above control levels for 10 μM DBT and about 13% for 5 μM DBT. Thus, the increases seen with TBT were far greater than those seen with DBT. Consistent with previous studies in rodent thymocytes and human T-cell leukemia cells, we saw an increase in cytosolic Ca^{2+}. However, we used much lower concentrations of TBT than were used in those studies (Chow et al., 1992; Stridh et al. 1999).

Exposure of NK cells to calcium ionophore, A23187, increased the phosphorylation of p44/42 and p38 at 5 min, but not at any other time point. In contrast, the concentration of TBT that caused the greatest increase in cytosolic Ca^{2+}, 500 nM, caused a similar increase in phospho-p44/42 as was seen with A23187 (about 2 fold), but this increase maintained out to 60 min as compared to only 5 min with A23187 (Table 1). 500 nM TBT caused no significant increase in phospho-p38 (Table 1) while A23187 caused an approximately 2.6 fold increase at 5 min. Furthermore, 300 nM TBT, which caused a much smaller increase in cytosolic Ca^{2+} than either A23187 or 500 nM TBT, caused much more significant increases in both phospho-p44/42 and phospho-p38. Thus, it appears that increases in cytosolic Ca^{2+} do not significantly account for the increases in MAPK activation that are seen with most TBT exposures. However, alterations of cytosolic Ca^{2+} concentration are important in the lytic response of NK cells, thus, the increases in cytosolic Ca^{2+} are likely to have important effects on NK cell function at the 500 nM and possibly the 300 nM exposures. TBT (300 nM) has been shown to suppress mitogenesis of peripheral blood mononuclear cells in both dolphins and humans (Nakata et al., 2002). It is possible that alteration of cytosolic Ca^{2+} levels seen with TBT could also contribute to this effect, if T cells were to show an increase in cytosolic Ca^{2+} levels as is seen in NK cells. This is quite possible as rat thymocytes show increases in cytosolic Ca^{2+} levels following TBT exposures (Chow et al., 1992)

In summary, the results indicate that BTs were able to increase cytosolic Ca^{2+} in NK cells, with TBT being much more effective than DBT. This increase in cytosolic Ca^{2+} appeared to be mainly due to an influx of Ca^{2+} from the extracellular compartment. The results also showed that increased Ca^{2+} could lead to a brief activation of the MAPKs, p44/42 and p38. Thus, it appears that the TBT-induced activation of MAPKs in NK cells cannot be fully accounted for by TBT-induced increases in cytosolic Ca^{2+}.

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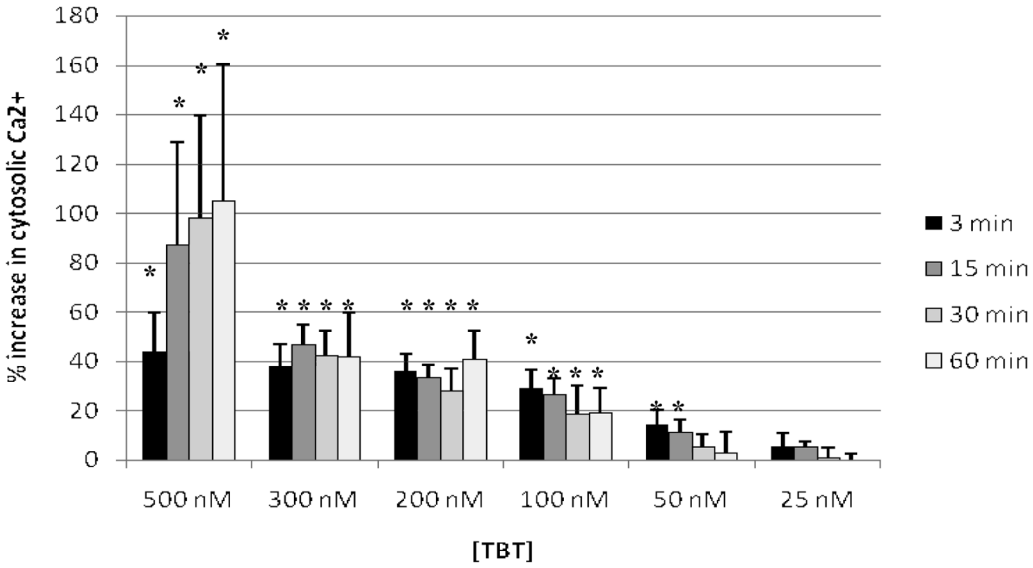

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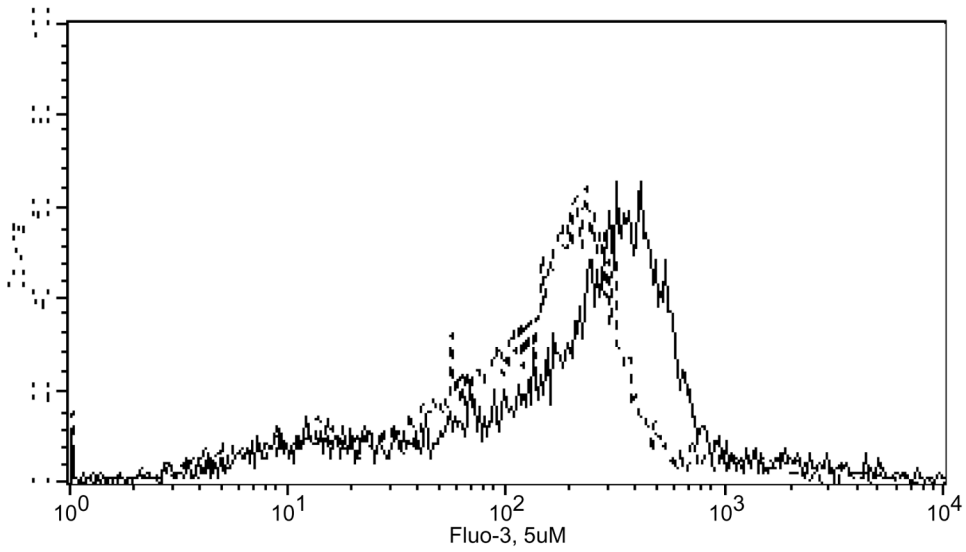

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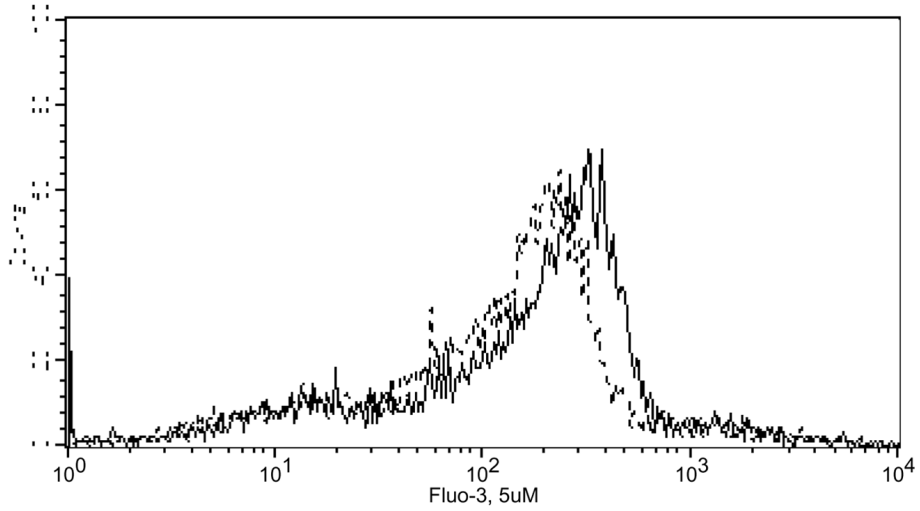

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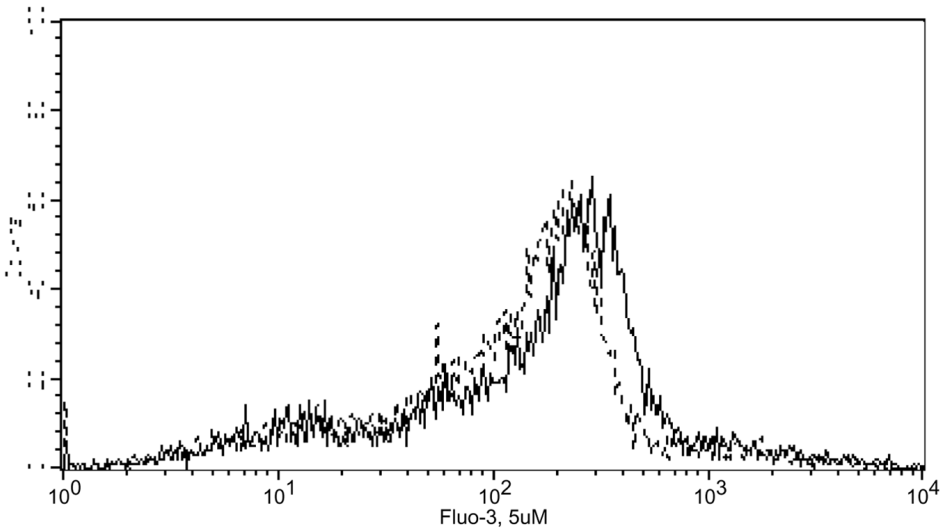


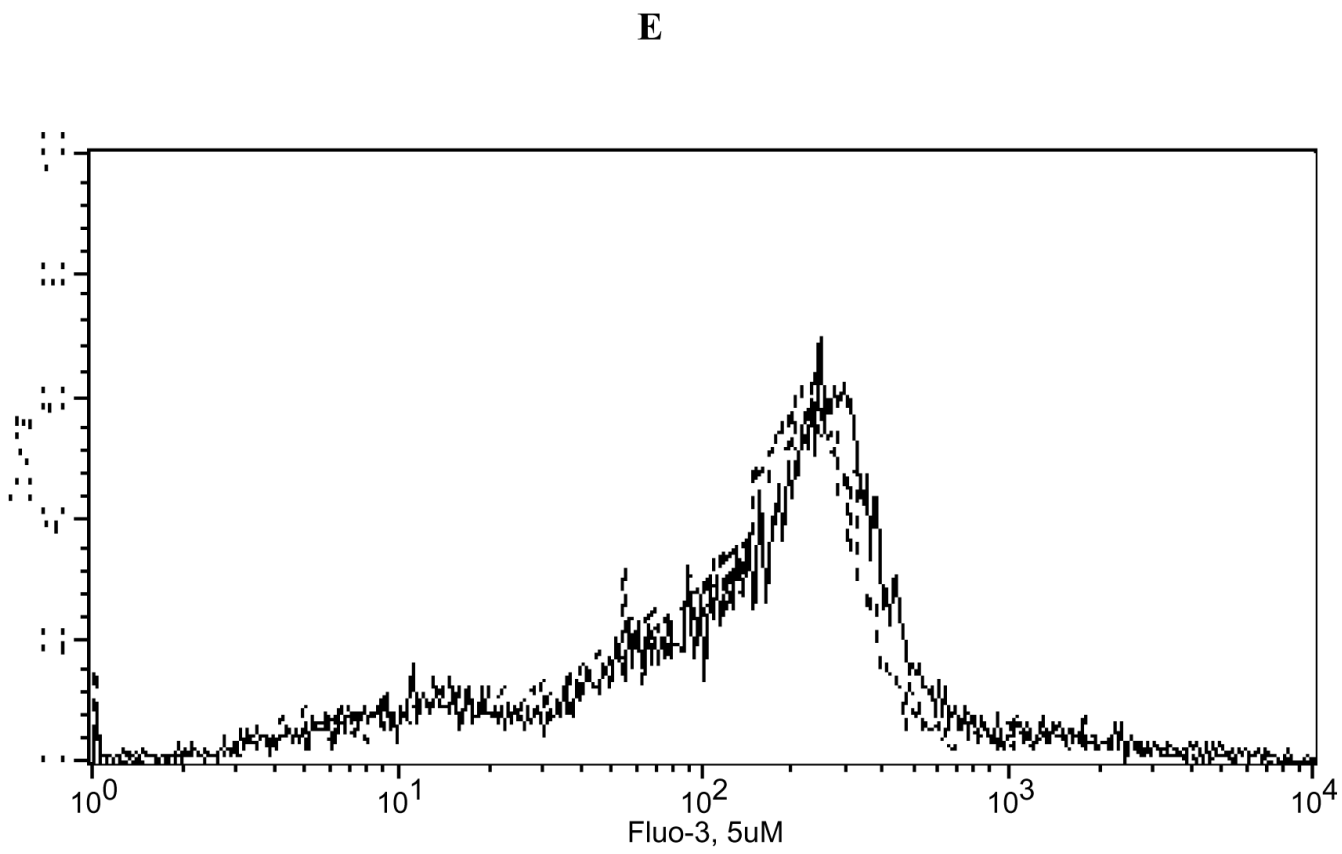
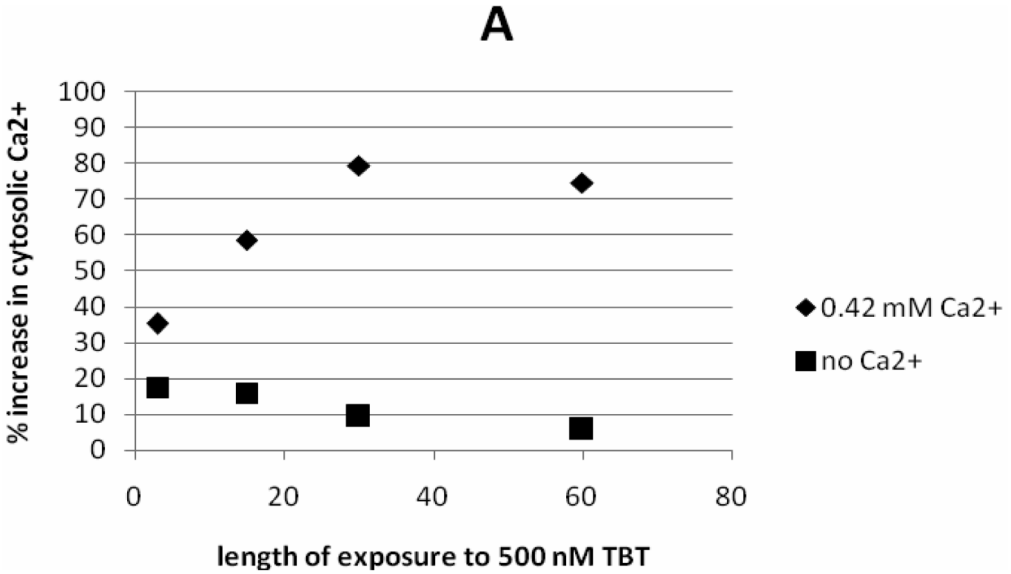
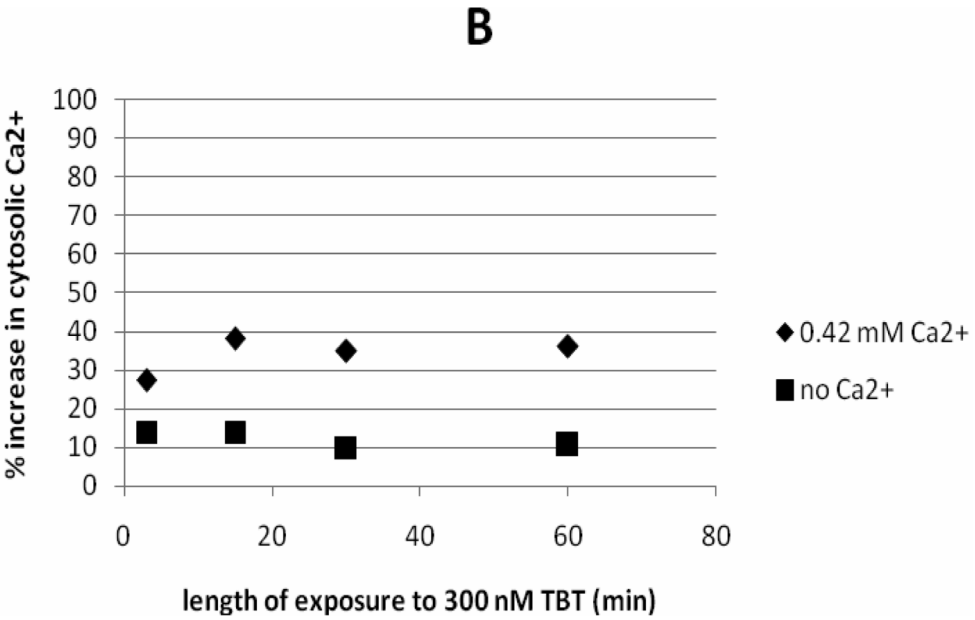


Figure 1.

Effect of exposure to TBT on cytosolic Ca^{2+} levels in NK cells. A.) 500-25 nM TBT for 3- 60 minutes. Values are percent increase in Fluo-3 mean fluorescence in treated cells as compared to cells that received no TBT exposure (mean ± S.D. of 4 separate determination using NK cells prepared from different donors (an asterisk * indicates a statistically significant difference ($P<0.05$)). B.) shift in mean fluorescence intensity of Fluo-3AM caused by a 30 min exposure of NK cells to 500 nM TBT from a representative experiment (dashed line = control cells; solid line = treated cells). C.) as described in (B) for a 30 min exposure of NK cells to 300 nM exposure to TBT. D.) as described in (B) for a 3 min exposure of NK cells to 200 nM exposure to TBT. E.) as described in (B) for a 3 min exposure of NK cells to 100 nM exposure to TBT.





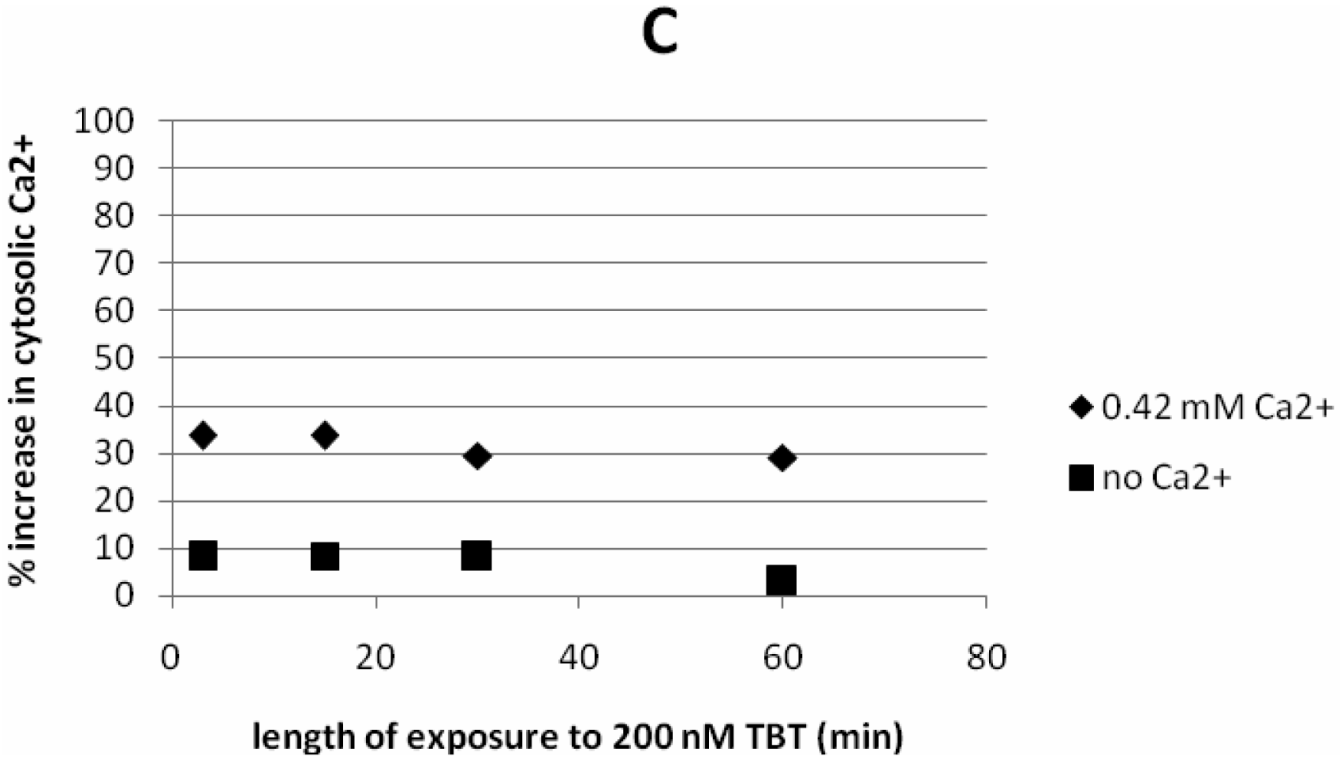
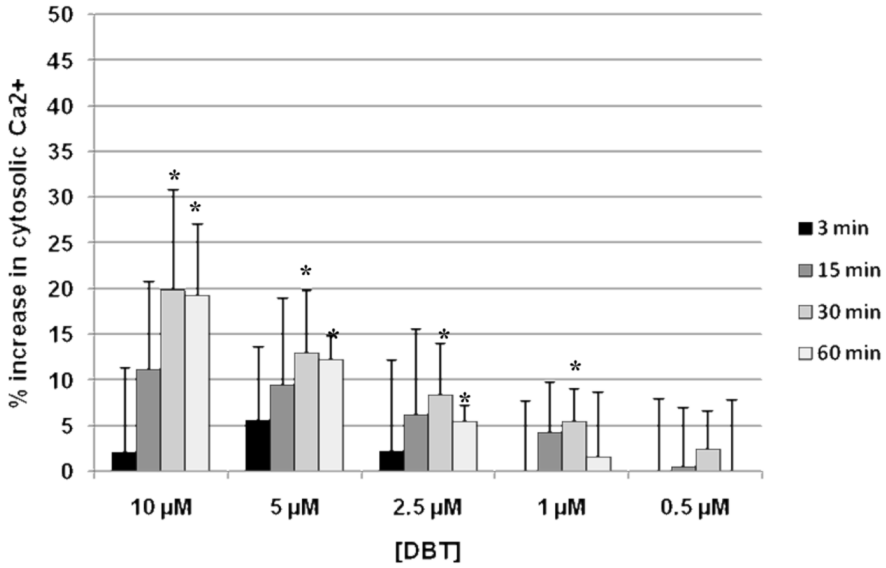


Figure 2.
Effect of exposure to TBT on cytosolic Ca^{2+} levels in NK cells in Ca^{2+} -co-containing versus Ca^{2+}-free media. A) increase in cytosolic Ca^{2+} seen with exposure to 500 nM TBT. B.) increase in cytosolic Ca^{2+} seen with exposure to 300 nM TBT. C.) increase in cytosolic Ca^{2+} seen 30 min exposure to 200 nM TBT.



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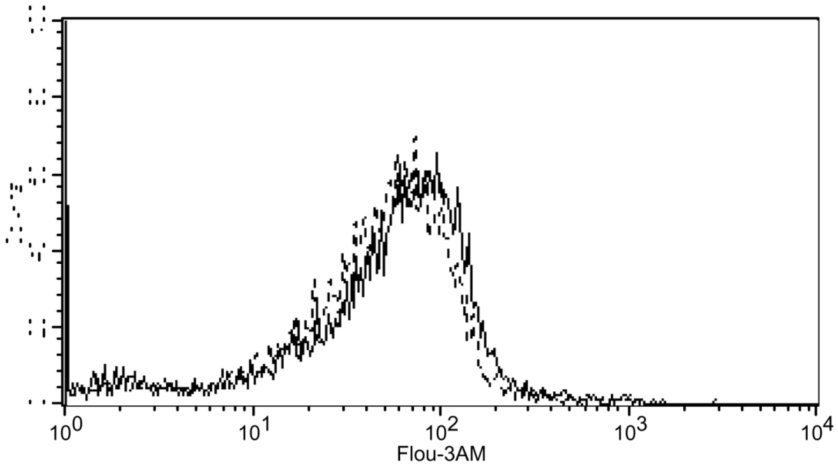


Figure 3.

Effect of exposure to DBT on cytosolic Ca^{2+} levels in NK cells. A.) 10-0.5 µM DBT for 3- 60 minutes. Values are the percent increase in Fluo-3 mean fluorescence in treated cells as compared to that in cells that received no DBT exposure (mean ± S.D. of 5 separate determination using NK cells prepared from different donors (an asterisk * indicates a statistically significant difference ($P<0.05$)). B.) Shift in mean fluorescence intensity of Fluo-3AM caused by a 30 min exposure of NK cells to 10 µM DBT from a representative experiment (dashed line = control cells; solid line = treated cells).

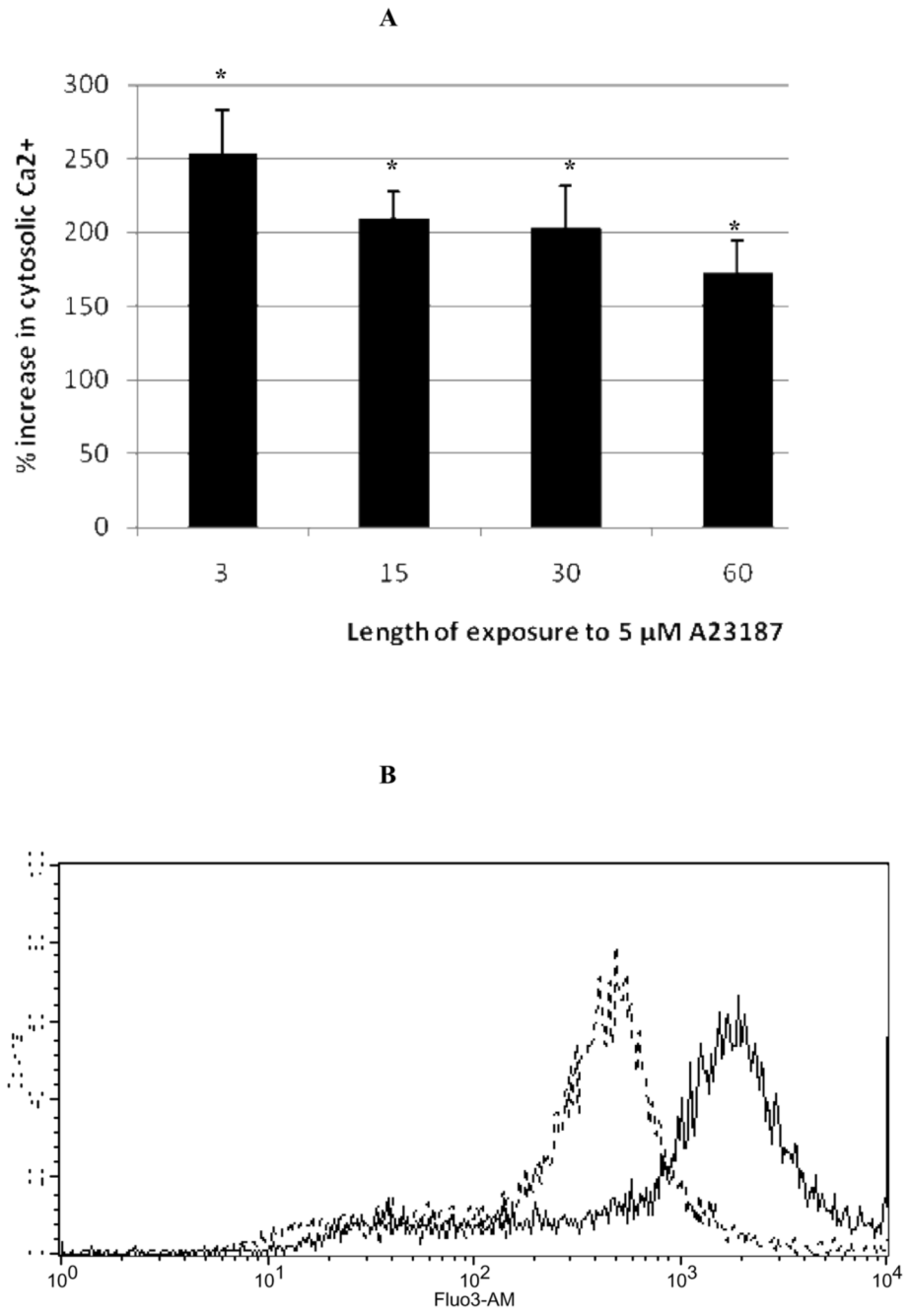
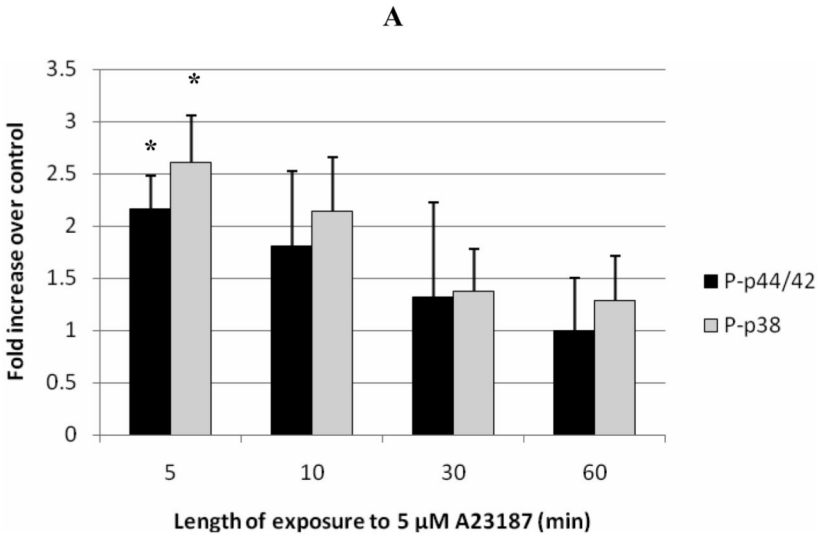


Figure 4.

Effect of exposure to calcium ionophore, A23187, on cytosolic Ca^{2+} levels in NK cells. A.) 5 µM A23187 for 3- 60 minutes. Values were determined by calculating the percent increase in Fluo-3 mean fluorescence in treated cells as compared to that in cells that received no A23187 exposure (mean ± S.D. of 3 separate determination using NK cells prepared from different donors (an asterisk * indicates a statistically significant difference ($P<0.05$)). B.) Shift in mean fluorescence intensity of Fluo-3AM caused by a 3 min exposure of NK cells to 5 µM A23187 from a representative experiment (dashed line = control cells; solid line = treated cells).



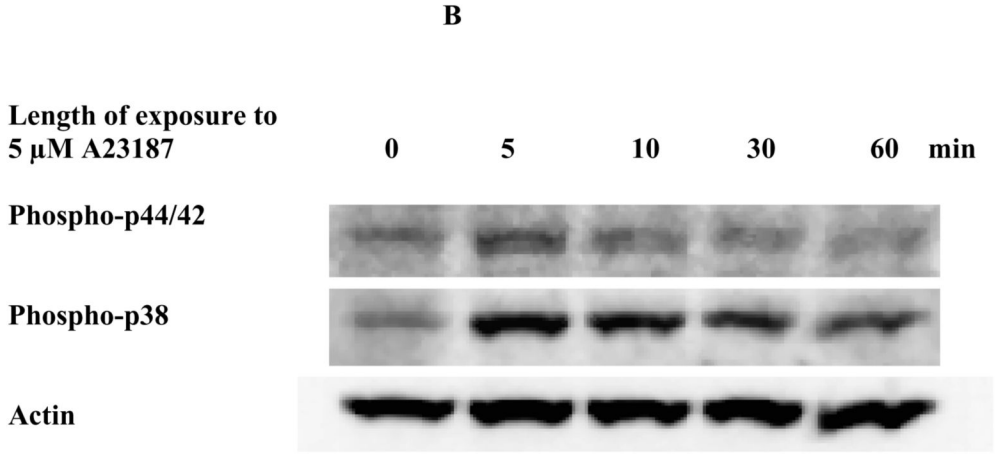


Figure 5.

Effect of 3-60 min exposure to 5 µM A23187 on the phosphorylation of the MAPKs, p44/42 and p38 in pure NK cells. A) phospho-p44/42 and phospho- p38 in NK cells exposed to 5 µM A23187 for 5-60 min. Values are fold increase in phosphorylation as compared to control NK cells (mean ± S.D. from three separate experiments using cells from different donors (an asterisk * indicates a statistically significant difference ($P<0.05$)). The density of each protein band was normalized to β-actin to correct for small differences in protein loading among the lanes. B) Representative Western blot of phospho-p44/42; phoshpho-p38 and actin.

Table 1

TBT-induced increases in phosphorylation of MAPKs, p44/42 and p38.[a]

	Length of exposure to TBT			
	5 min	10 min	30 min	60 min
Fold increase in Phospho-p44/42 (500 nM TBT)	2.6	2.5	2.7	2.7
Fold increase in Phospho-p38 (500 nM TBT)	NS[b]	NS	NS	NS
Fold increase in Phospho-p44/42 (300 nM TBT)	2.8	3.5	4.1	5.0
Fold increase in Phospho-p38 (300 nM TBT)	2.1	2.2	2.3	2.0

[a] from Aluoch and Whalen, 2005

[b] NS=not significant