An *in vitro* Study of Drug-induced Degranulation of Human HMC-1 Mast Cells and Rat RBL-2H3 Cells

by

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ABSTRACT

Mast cells and basophils participate in biological responses such as inflammation and allergic reactions and contain potent mediators such as histamine and cytokines. Some drugs, for example opioids, are known to trigger mast cell degranulation with the release of immunogenic mediators that may cause severe side effects.

The aim of this study was to investigate if the human mast cell line HMC-1 is a useful model to study codeine-induced degranulation *in vitro*. The rat basophilic leukemia RBL-2H3 cell line was used as a reference. Time- and concentration-dependent effects of codeine, compound 48/80, and A23187 on the degranulation of HMC-1 cells and RBL-2H3 cells were investigated by measuring extracellular levels of β-hexosaminidase and [^{3}H]serotonin.

Neither codeine nor compound 48/80 produced any significant basophil or mast cell degranulation, whereas the calcium ionophore A23187 triggered a degranulation of both cell lines.

In conclusion, none of the cell lines are useful as relevant, robust and reproducible *in vitro* models to study drug-induced mast cell degranulation.

INTRODUCTION

Mast cells are derived from haematopoietic progenitor cells and have a crucial role in early recognition of pathogens (Urb and Sheppard 2012). They are best known for their adverse effects associated with allergic reactions (Veien *et al.,* 2000). When mast cells are activated, for example in allergic reactions, large amounts of granule-stored inflammatory mediators such as histamine, proteases, and cytokines are released (Buku *et al.,* 2008). In the clinical situation, hypersensitivities due to mast cell degranulation are manifested as urticaria, skin rashes, and in extreme cases anaphylactic shock which potentially can be life threatening (Golembiewski, 2002). Some drugs, for example opioids and opiates that are therapeutically used for its analgesic effects, may cause anaphylaxis by a mechanism that directly triggers mast cell degranulation (Sheen *et al.,* 2007). The opiates codeine and morphine have been found to be more prone to induce mast cell degranulation than other opioids. Studies have suggested that the mechanism behind opioid-induced activation and degranulation of mast cells is mediated by immunoglobulin (Ig) E whereas other studies have shown that the activation of mast cells by codeine is independent of both IgE and the high-affinity IgE receptor FcεRI (Sheen *et al.,* 2007). FcεRI is necessary for the induction of IgE-mediated allergic reactions and IgE is a potent stimulus for mast cell mediator release when bound to the FcεRI receptors (Toru *et al.,* 1996). Cross-linking of FcεRI expressed on mast cells causes the release of various inflammatory mediators, which trigger allergic reactions (Facci *et al.,* 1995). There are two other known pathways that stimulate degranulation of mast cells; direct G-protein activation and activation through an opioid receptor. When the different pathways are activated is currently unknown (Sheen *et al*., 2007).

Hence, the mechanism behind drug-induced mast cell degranulation and the onset of the anaphylactic states is not fully understood, and in order to prevent drug-induced anaphylaxis more research is needed. For that purpose, a relevant and reproducible *in vitro* model of drug-induced human mast cell degranulation is required.

In the present study the usefulness of the human mast cell line HMC-1 as a model to study opioid-induced mast cell degranulation *in vitro* was investigated. The HMC-1 cell line originates from a patient with mast cell leukemia (Butterfield *et al.,* 1988), and has

shown several characteristics of tissue mast cells, including the expression of histamine, tryptase, and heparin (Nilsson *et al.*, 1994). Therefore this cell line has been widely used to study the biology of mast cell function *in vitro*. The results of this study have been compared to those obtained using the rat basophilic leukemia cell line RBL-2H3, originally isolated from a rat treated with a potent carcinogenic compound that produced a granulocytic leukemia with peripheral blood basophilia (Leonard *et al.*, 1971). Due to the ability of RBL-2H3 cells to release histamine in an IgE-dependent manner and the expression of high-affinity FcεRI receptors, the RBL-2H3 cell line has been considered to model mast cells and has therefore been used extensively and successfully to study IgE-dependent degranulation (Passante *et al.*, 2009a).

There are numerous methods available for measuring exocytosis of mediators from secretory granules as indication of mast cell degranulation. A common, rapid, and inexpensive approach is to assay enzymes released from the mast cells into the cell supernatant during degranulation, such as tryptase (Butterfield *et al*., 1990) or β-hexosaminidase. It has been found that β-hexosaminidase is released from the granules together with for example histamine, and that the enzyme is easily detected by using a simple enzyme-substrate reaction in a microplate assay format (Safaralizadeh *et al.*, 2009).

Since both histamine and serotonin are present in human and rodent mast cell secretory granules, another common method to study mast cell degranulation is to measure the release of tritium-labeled ([^{3}H])serotonin from mast cells (Ritzén 1967). Mast cells and basophils have a selective uptake of serotonin, and the [^{3}H]serotonin is released from the cells upon stimulators such as compound 48/80 and other drugs (Ferjan and Lipnik-Štangelj 2013).

The aim of this study was to examine whether the HMC-1 cell line was a suitable model and if specific methods were useful to study degranulation induced by codeine.
In this study, the time- and concentration-dependent effects of codeine, compound 48/80, and the calcium ionophore A23187 on the degranulation of HMC-1 cells and RBL-2H3 cells have been investigated by measuring the extracellular activity of β-hexosaminidase and the release of [^{3}H]serotonin. Both compound 48/80 and A23187 were used as positive controls. Furthermore, the importance of IgE molecules in the

opioid-induced activation and degranulation of mast cells have been studied by pre-incubating the HMC-1 cells and the RBL-2H3 cells with monoclonal anti-DNP IgE.

MATERIALS & METHODS

Chemicals

4-Nitrophenyl *N*-acetyl-β-D-glucosaminide (chromogenic substrate for the β-hexosaminidase analysis), codeine-d_3 solution, compound 48/80, the calcium ionophore A23187, and monoclonal anti-dinitrophenyl antibodies produced in mouse (anti-DNP IgE) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DNP-BSA (albumin from bovine serum, 2,4-dinitrophenylated) was purchased from Molecular Probes/Life Technologies (Sweden). The chromogenic substrate was dissolved and diluted in 0.2 M citrate buffer pH 4.5. The codeine was dissolved in methanol (1 mg/ml). Compound 48/80 was dissolved in distilled water (10 mg/ml) and further diluted in the culture media. A23187 was dissolved in ethanol to a concentration of 9.55 mM and further diluted in the culture media.

Cell cultures

Rat basophilic leukaemia RBL-2H3 cell line (passage range 15-41) was obtained from American Type Culture Collection (Manassas, VA, USA). RBL-2H3 cells were cultured in Eagle's minimum essential medium (MEM) with Earl´s salts, supplemented with 15 % fetal bovine serum (FBS), 100 U/ml penicillin + 100 µg/ml streptomycin (1 % PEST). The culture medium was changed every other day and the cells were passaged twice a week. The human mast cell line HMC-1 (passage range 8-18) was a kind gift from Gunnar Nilsson, Centrum för allmänmedicin, Karolinska Institutet, Stockholm, with a material transfer agreement (MTA) from Mayo Clinic Ventures, Joseph H. Butterfield, MD. The HMC-1 cells were cultured in IMDM supplemented with 10 % FBS, 1 % PEST, 2 mM L-glutamine and 1.2 mM 1-thioglycerol. Every week, the cells were passaged and after 3-4 days, the culture medium was changed. Both cell types were cultured in 75 cm^2 cell culture flasks at 37 ºC in humidified atmospheric pressure with 5 % CO_2.

β-hexosaminidase assay

To analyze the β-hexosaminidase activity, the method of Granberg *et al*. (2001) was used. Briefly, 50 µl samples were added to a 96-well microtiter plate and 50 µl of 2 mM *p*-nitrophenyl-*N*-acetyl-D-glucoseaminide in 0.2 M citrate-buffer was added to start the reaction. The plates were incubated for two hours at 37 ºC and the reaction was stopped by adding 150 µl 1 M Tris-buffer (pH 9.0). The optical density was measured at 405 nm in a computer-operated SpectrostarNano microplate spectrophotometer reader (BMG Labtech, Germany). Triton X-100 was added (to a final concentration of 1 %) to 4-6 of the wells on each microtiter plate and used to determine the total cellular content of the enzyme. The release is expressed as a percentage of total cellular content.

Release of [^{3}H]serotonin

The RBL-2H3 cells were plated in 24-well plates at a density of 0.86 x 10^6 cells/ml and incubated for approximately 18 hours with 50 µl of 0.1 µCi/ml [^{3}H]serotonin in each well. The wells were washed two times with PBS prior to addition of 350 µl MEM medium (w/o phenol red) and 50 µl of each of the test compounds. The plates were incubated at 37 ºC for 30 minutes followed by centrifugation for 10 minutes at 300 *g*. The HMC-1 cells were seeded in T-75 culturing flasks and incubated for approximately 18 hours with a [^{3}H]serotonin concentration of 0.1 µCi/ml. The cells were transferred into 24-well plates at a concentration of 0.86 x 10^6 cells/ml. The test compounds were added (50 µl) and the plates were incubated for 10 minutes at 37 ºC and thereafter centrifuged for 10 minutes at 300 *g*. Aliquots of 250 µl were analyzed for [^{3}H]serotonin radioactivity by liquid scintillation counting with quench correction and the tritium release was calculated and compared to the radioactivity of the supernatant and cell lysate.

IgE-dependent degranulation

The RBL-2H3 cells were cultured in 24-well plates at a density of 5 x 10^5 cells/ml and incubated for 24 hours before addition of 10 µl anti-DNP IgE final concentration of 5 ng/ml. The plates were incubated for 1 hour at 37 ºC (in order to sensitize the cells) and then washed twice with PBS. The HMC-1 cells were incubated with anti-DNP IgE in culture medium (final concentration of 5 ng/ml) in 15 ml falcon tubes for one hour at 37

ºC, and washed twice with PBS before transferred into 24-well plates to a concentration of 5.7 x 10^5 cells/ml.

After adding 350 µl assay medium w/o phenol red, 50 µl assay medium containing the test compounds or the antigen DNP-BSA (final concentration of 50 ng/ml) was added to the both cell lines. The cells were incubated for 30 min at 37 ºC and after centrifugation for 10 min at 300 *g*, 50 µl aliquots per well were analysed for β-hexosaminidase activity.

Statistical analyses

All statistical analyses were performed using one-way ANOVA with post-hoc Dunnett's multiple comparisons test, or where appropriate, Student's *t*-test (GraphPad Prism 5 for Mac OS X, GraphPad Software Inc., San Diego, CA, USA), and a *p* value of 0.05 (or less) was considered statistically significant. All data are presented as the means ± SEM of at least three separate experiments.

Literature search

Articles were searched in PubMed (http://www.ncbi.nlm.nih.gov/pubmed) by using the following search terms: anaphylactic shock, anaphylaxis, codeine, human mast cells, HMC-1, RBL-2H3, compound 48/80, A23187, hexosaminidase, degranulation, opioides, serotonin, histamine, IgE receptor. These words and terms were combined and related articles were also used. Boolean operators such as AND, OR and NOT were used.

Ethical considerations

Commercially available cell line RBL-2H3 from rat and the established and published cell line HMC-1 from human were used. Ethical issues regarding the use of human cells have to be taken into consideration. However, the cells were harvested with consent, are already established and have been used in experiments since the 1980s and therefore we believe that there are no ethical issues relevant to the present study.

RESULTS

The concentration-dependent effects of codeine upon degranulation of β-hexosaminidase from human HMC-1 mast cells and rat RBL-2H3 basophils are shown in Figure 1. Under the culture conditions used in this study, neither codeine nor compound 48/80 (used as a positive control) produced any statistically significant release of β-hexosaminidase from the cell lines. Regardless of treatment, the extracellular levels of the enzyme remained between 7.3-9.1 % of total cellular content in the RBL-2H3 cell cultures and between 3.8-5.1 % in the HMC-1 cultures. The calcium ionophore A23187, on the other hand, induced a significant degranulation of 61 ± 7 % in the RBL-2H3 cells (Fig. 1B; $p < 0.0001$), but the A23187-induced degranulation in the HMC-1 cells was modest in comparison (Fig. 1D; from 4.6 ± 0.2 % in untreated controls to 7.9 ± 0.5 %; $p< 0.0001$). Solvents, such as ethanol or methanol, may affect the degranulation processes even at low concentrations. In this study, the final solvent concentration never exceeded 1 %, and no major effect on the degranulation could be observed.

The [^{3}H]serotonin and β-hexosaminidase release from HMC-1 and RBL-2H3 cells are shown in Figure 2. Codeine and compound 48/80 had no stimulatory effect on the release of β-hexosaminidase or [^{3}H]serotonin in the cell lines (Fig. 2). Actually, codeine at a concentration of 0.1 µg/ml and 0.3 µg/ml produced a small but statistically significant decrease in the β-hexosaminidase activity in RBL-2H3 cells (from 7.8 ± 0.2 % in untreated controls to 5.5 ± 0.9 % and 5.5 ± 0.1 %, respectively; $p< 0.01$), which in this study probably is not biologically significant.

A23187 produce a massive degranulation in RBL-2H3 cells loaded with [^{3}H]serotonin (72-94 %), but not in HMC-1 cells (Fig. 2C and 2F, respectively).

On average, the extracellular levels of [^{3}H]serotonin were high in the HMC-1 cells compared to the RBL-2H3 cells. In the untreated control wells, the extracellular levels of [^{3}H]serotonin were approximately 86 % of the Triton X-100-treated wells, compared to 19 % in the RBL-2H3 cell line. This indicates that the HMC-1 cells are not as efficient as the RBL-2H3 cells to accumulate and/or retain serotonin, giving a high basal level of radioactivity.

In Figure 3, the β-hexosaminidase release from HMC-1 and RBL-2H3 cells after pre-incubation with anti-DNP IgE, prior to exposure to the test compounds, is presented. Similar results as from the experiments above were obtained, with no apparent effect of codeine or compound 48/80, whereas A23187 produced a major degranulation of β-hexosaminidase in the RBL-2H3 cells and a small but statistically significant degranulation in the HMC-1 cells (Fig. 3). The antigen DNP-BSA was also without effect in the IgE-sensitized cells.

DISCUSSION

A relevant, robust and reproducible human *in vitro* model of mast cell degranulation is important to study the involvement of mast cell in drug-induced anaphylaxis. In the present study, the effects of codeine, A23187 and compound 48/80 upon the degranulation of the β-hexosaminidase and [^{3}H]serotonin have been studied in human HMC-1 cells and rat RBL-2H3 cells. The results suggest that none of the cell lines are useful for the aimed purpose, due to their weak degranulation response to drugs known to potently induce mast cell degranulation, in this case codeine.

The HMC-1 cell line is derived from a mast cell leukaemia patient, and is one of the human cell lines available that grows continuously and expresses a phenotype similar to human mast cells found *in vivo* (Nilsson *et al.,* 1994). The HMC-1 mast cell line has been widely used in *in vitro* studies (Sundström *et al.,* 2003), but is limited in its applications because of a small expression of the human IgE receptor FcεRI (Xia *et al.,* 2011).

The RBL-2H3 rat cell line is a cloned leukaemia cell line isolated from rat. The RBL-2H3 cell line has been used in numerous studies to investigate IgE–FcεRI interactions and how this interaction is involved in the degranulation process (Smith *et al.,* 1997).

Previous studies *in vivo* have shown that the mast cell degranulation is induced by the opiate codeine (Scherer *et al.,* 2007) and codeine has been used as a positive control in skin prick tests (Yoo *et al.,* 2014). However, there are differences between *in vivo* and *in vitro* studies which might affect results associated with mast cell degranulation. For

example, different subtypes of mast cells may be activated by codeine (Sheen *et al*., 2007).

Mast cells can be activated by immunological (IgE) and by non-immunological (G-protein or opioid receptor) pathways. These pathways lead to the release of mediators such as histamine (Sheen *et al.,* 2007). The results from Blunk *et al*. (2004) indicate that the activation of skin mast cells by codeine depends on the direct activation of G-proteins on the mast cells. Our results show no codeine-induced release of either β-hexosaminidase, or [^{3}H]serotonin, from the two examined cell lines. We can only speculate on the reason for this lack of degranulation response under the experimental conditions used, mainly due to the fact that the mechanism behind codeine-induced mast cell degranulation has not been fully understood. Possible speculative reasons for absence of codeine induced degranulation may be lack of opioid receptors, FcεRI receptors, G-protein or granulae in cytoplasm.

The effects of synthetic compound 48/80 were also examined, since earlier studies have shown that this compound is a very potent inducer of mast cell degranulation (Swieter *et al.,* 1993). However, it seems that *in vitro,* compound 48/80 is able to stimulate only certain subtypes of mast cells to induce the release of inflammatory mediators (Swieter *et al.,* 1993). Earlier studies have shown that RBL-2H3 cells are not activated by the compound (Lim *et al.,* 2010). This is supported by our findings that compound 48/80 (in the concentration range of 1.5 µg/ml -15 µg/ml) was without effect in the RBL-2H3 cells, as measured by the release of β-hexosaminidase and [^{3}H]serotonin. On the other hand, according to the report of Swieter *et al*. (1993) there was a significant degranulation of histamine from the RBL-2H3 cells when they were grown in co-cultures with fibroblasts when stimulated by compound 48/80 (in the concentrations range of 0.1 µg/ml – 10 µg/ml).

Regarding the HMC-1 cells, compound 48/80 (in the concentration range of 1.5 µg/ml - 15 µg/ml) did not provoke release of neither β-hexosaminidase or [^{3}H]serotonin. Previous studies have shown that the compound 48/80 displayed a strong ability of evoking β-hexosaminidase (Lee *et al*., 2010) and histamine release (Nazarov and Pronina, 2012). The discrepancy between these findings and our results may be due to different methods to study and analyse the degranulation process, e.g. the use of

fluorescence or absorbance, or differences in concentrations of the compound used (a maximal concentration of 15 µg/ml in the present study and 20 µg/ml in the study of Nazarov and Pronina).

Under normal conditions, the RBL-2H3 cells respond to calcium ionophores, such as ionomycin (Fowler *et al.*, 2003) or A23187 (Passante *et al.*, 2009b), by increasing the intracellular calcium levels and thereby evoking mast cell degranulation. The treatment of both HMC-1 cells (Balletta *et al.*, 2013) and RBL-2H3 cells (Artalejo *et al.*, 1998), with the calcium ionophore A23187 have shown to induce degranulation, as measured by the release of β-hexosaminidase. This is supported by the results in the present study, where especially the RBL-2H3 cells responded with a major release of both β-hexosaminidase and [^{3}H]serotonin when incubated with A23187.

In the current study, the antigen DNP-BSA was without effect in the IgE-sensitized cells. Earlier studies indicate that IgE-sensitized RBL-2H3 cells are activated by antigen (Morita and Siraganian 1981). The lack of IgE-mediated degranulation of HMC-1 and RBL-2H3 cells might be because of absence of connection between antibody and cell surface or between antigen and antibody. Another reason may be different types of IgE antibodies and antigen used.

In conclusion, the present study was not able to confirm that codeine or compound 48/80 induces degranulation of HMC-1 cells or RBL-2H3 cells. Thus, these cell lines are not useful as *in vitro* models for opioid-induced activation and degranulation of mast cells. Further studies are required to develop relevant human *in vitro* models to investigate drug-induced degranulation of mast cells in order to find new therapeutic strategies to prevent allergic reactions such as anaphylactic shock in the clinical setting.

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FIGURES WITH LEGENDS

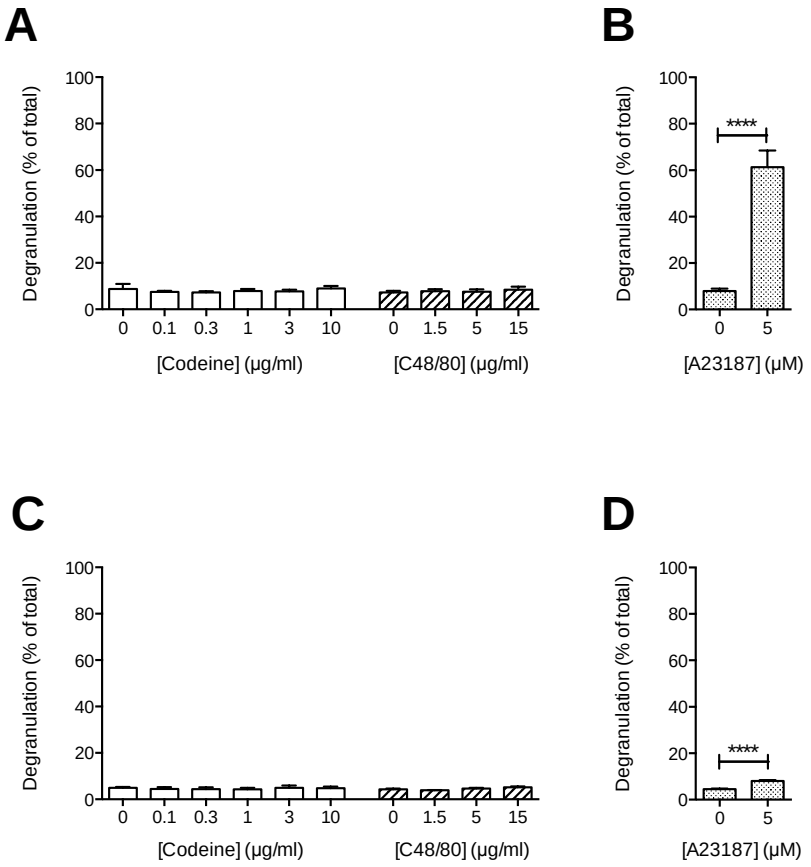

Fig. 1. Effects of (**A, C**) codeine, compound 48/80 and (**B, D**) A23187 on the degranulation of (**A, B**) RBL-2H3 cells and (**C, D**) HMC-1 cells, as measured by extracellular β-hexosaminidase activity. Data are means ± SEM of 4-8 separate experiments, and expressed as percentage of maximum enzyme activity (produced by incubating parallel wells with the detergent Triton X-100). Statistically significant differences (Student's *t*-test) are indicated as ****$p < 0.0001$, when compared with corresponding untreated control wells.

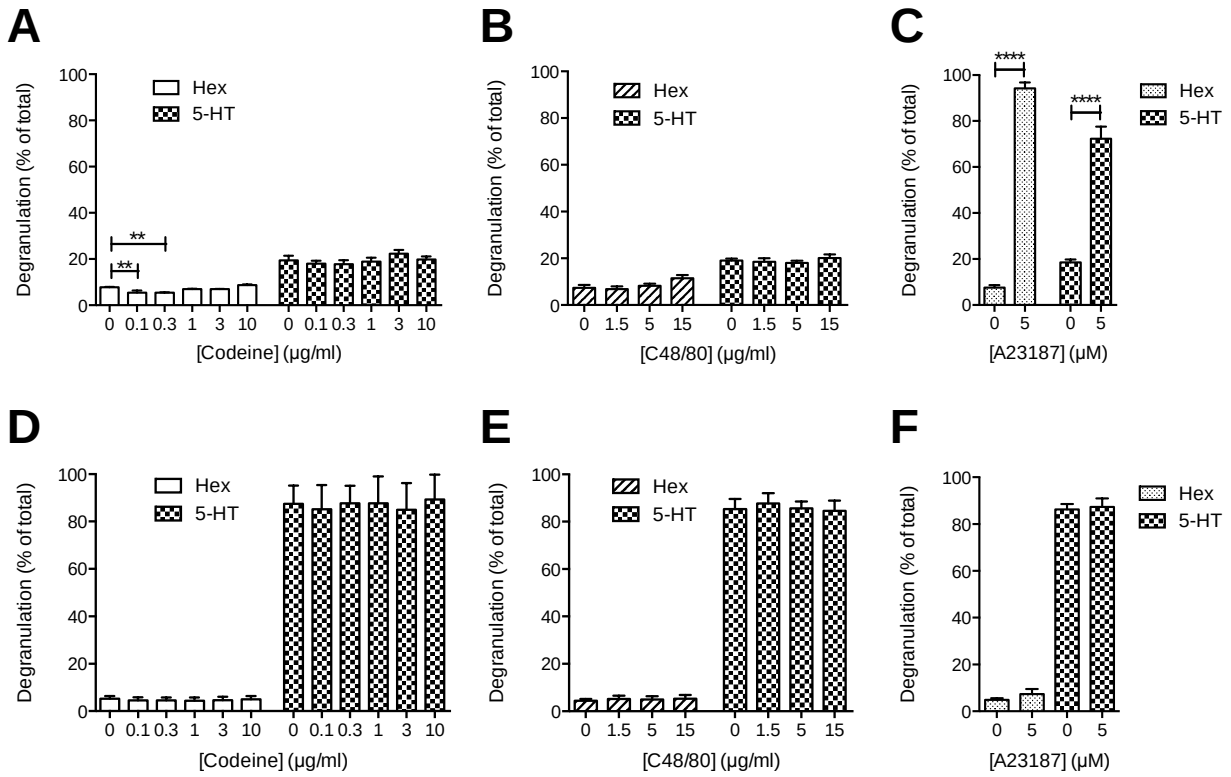

Fig. 2. Effects of (**A, D**) codeine, (**B, E**) compound 48/80, and (**C, F**) A23187 on the degranulation of (**A-C**) RBL-2H3 cells and (**D-F**) HMC-1 cells, as measured by extracellular β-hexosaminidase activity (Hex.) and release of [^{3}H]serotonin (5-HT). Data are means ± SEM of 3-6 individual experiments, and expressed as percentage of maximum enzyme activity or total [^{3}H]serotonin content (obtained by incubating parallel wells with Triton X-100). Statistically significant differences (using one-way ANOVA with Dunnett's multiple comparisons test [in panel **A**], or Student's *t*-test [in panel **C**]) are indicated as $^{**}p < 0.01$, and $^{****}p < 0.0001$, when compared with corresponding untreated control wells.

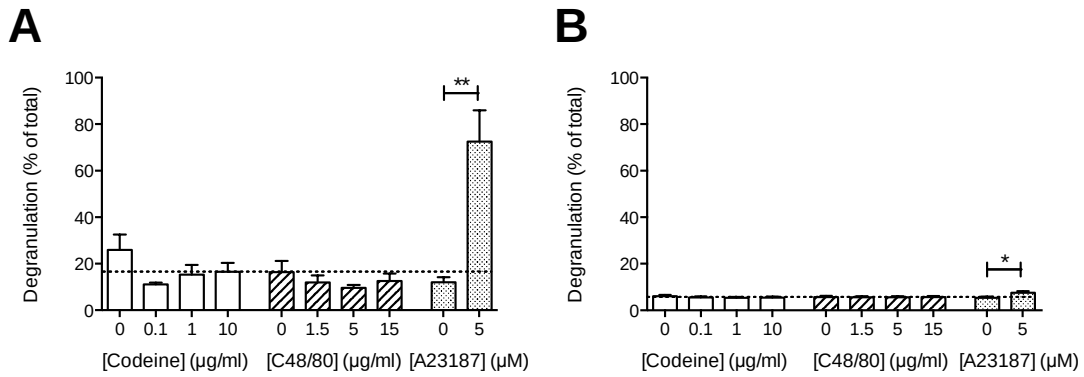

Fig. 3. Effects of codeine, compound 48/80 and A23187 on the degranulation of β-hexosaminidase from (**A**) RBL-2H3 cells and (**B**) HMC-1 cells pre-incubated for one hour with anti-DNP IgE-containing medium. Dotted line indicates extracellular β-hexosaminidase activity in parallel wells exposed to 50 ng/ml of the antigen DNP-BSA. Data are means ± SEM of 4-6 individual experiments, and expressed as percentage of maximum enzyme activity. Statistically significant differences (using Student's *t*-test) are indicated as $*p < 0.05$, and $**p < 0.01$, when compared with corresponding untreated control wells.