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Anti-allergic cromones inhibit neutrophil recruitment onto vascular endothelium *via* annexin-A1 mobilization

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Abstract

Objectives—The anti-allergic 'mast cell stabilising' cromones release the anti-inflammatory protein annexin A1 (Anx-A1) from U937 cells. These drugs also inhibit polymorphonuclear leukocyte (PMN) trafficking. Since PMN can synthesise and release large amounts of Anx-A1, we hypothesised that a similar mechanism could operate upon application of cromones to these cells.

Methods and Results—Intravital microscopy was used to monitor the actions of cromones in the inflamed microcirculation. Reperfusion injury provoked a dramatic rise in adherent and emigrated leukocytes in the mesenteric vascular bed, associated with augmented tissue levels of myeloperoxidase (MPO). Nedocromil (2-20mg/kg) significantly ($p<0.05$) inhibited cell adhesion and emigration, as well as MPO release, in wild type but not Anx-A1$^{-/-}$ mice. Short pre-treatment of human PMN with nedocromil (10nM) inhibited cell adhesion ($p<0.05$) in the flow chamber assay, and this effect was reversed by a specific anti-AnxA1 or combination of anti-FPR1 and anti-FPR2 - but not an irrelevant control - antibodies. Western blotting experiments revealed that cromones stimulate PKC-dependent phosphorylation and release of Anx-A1 in human PMN.

Conclusions—We propose a novel mechanism to explain the anti-inflammatory actions of cromones on PMN trafficking, an effect that has long puzzled investigators.

Keywords

Nedocromil; cromoglycate; PKC; inflammation; FPR receptors

Cromones are a group of anti-allergic drugs of which sodium cromoglycate and sodium nedocromil are the exemplars. Early studies on the mechanism of action of these mast cell

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stabilisers suggested that they blocked mast cell degranulation, hence mediator release, when this triggered by various stimuli including IgE [1] . However these drugs can affect other facets of the inflammatory process including inhibition of eicosanoid [2] and cytokine [3] release.

Leukocyte emigration [4] is also inhibited by these drugs and, for instance, cromoglycate and ketotifen exhibit a protective role in neutrophil-dependent pathologies including intestinal [5] and pulmonary[6] ischaemia-reperfusion (IR) model. Mast cells, located in close apposition to the vessels, are an important source of pro-inflammatory mediators released at the site of inflammation[7]. Mast-cell derived mediators activate adhesion molecules from the local environment leading to leukocyte recruitment ([7]) utilising a well-characterised multi step mechanism (reviewed in [8]). Clearly inhibition of the release of mediators from these mast cells could explain the effect of the cromones on PMN trafficking. However, other work points to a separate, direct, effect of these compounds on the PMN [9].

Anx-A1 is a 37kDa glucocorticoid (GC)-regulated protein that mimics their effects in several in vivo and in vitro model systems [10-12]. GCs not only induce the Anx-A1 gene in many cells but also increase secretion of the protein from existing intracellular pools by stimulating PKC activity[13]. Once secreted the protein acts in a paracrine/autocrine fashion, utilising formyl peptide receptors (FPR) [14, 15] to produce its biological effects. The N-terminal region of Anx-A1 bears the biological activity of the protein and phosphorylation on Ser^{27} residue is crucial for protein export and secretion [13]. While studying the rapid mechanism of action of GC in a pro-monocytic U937 cell line, we noted that cromones promoted the rapid release of Anx-A1[16]. This occurred through inhibition of phosphatase PP2A, that constitutively limits PKC activity thereby potentiating the phosphorylation and release of Anx-A1 [16].

Anx-A1 is found in high amounts in circulating neutrophils and monocytes [17], from which the protein is externalized and cleaved after leukocyte adhesion to the endothelium in vitro [18]. The protein strongly inhibits neutrophil recruitment in vivo [19] and in vitro [20, 21] . In this study, we sought to investigate the mechanism of action of cromones on PMN emigration.

Material & Methods

Two distinct, yet complementary models were used. In an *in vivo* reperfusion injury model using Anx-A1$^{-/-}$ and an *in vitro* flow chamber assay using human cells together with a specific neutralizing Anti-Anx-A1 antibody. We have also assessed the effect of the cromones on the internalisation of FPR1 and FPR2 by FACs analysis and in the flow chamber assay. Cromones were also tested in zymosan-induced peritonitis, an acute model of inflammation. A full description of the methods used is available in the Supplemental Materials.

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Results

Alterations in the mouse mesenteric microcirculation following IR and the effect of nedocromil

In this model of IR in the mesenteric circulation, clamping and reopening of the superior mesenteric artery (SMA) induced a significant increase in the number of adherent and emigrated cells observed microscopically in WT mice, when compared to the sham operated animals (Figure 1A). This activation of the inflammatory process within the post-capillary venule endothelium is attenuated by the administration of (2mg/kg) nedocromil given 45 min pre-reperfusion.

We then investigated the mechanism by which nedocromil exerts this protective effect. The IR protocol induced a reduction in V_{WBC} (rolling velocity) in WT mice, which was associated with a significant increase in the degree of cell adhesion and emigration, as assessed 45 min after reperfusion (Figure 1B). Treatment of mice with a low (2mg/kg) dose of nedocromil strongly inhibited cell adhesion and emigration but had no effect on V_{WBC}. At the higher dose of 20mg/kg, nedocromil inhibited all three parameters under observation-cell rolling velocity, adhesion and emigration - although the latter did not reach statistical significance (Figure 1B).

We next tested the potential involvement of Anx-A1 in the observed intravascular effects of nedocromil using Anx-A1$^{-/-}$ mice. In these animals, the IR procedure did not alter V_{WBC} but produced the expected increase in cell adhesion and emigration. Within the time frame of these experiments, no difference between the genotypes observed with respect to the cellular response in the microcirculation (Figure 1B). However, nedocromil (2 mg/kg or 20 mg/kg) was without any discernable effect on the leukocyte-endothelium interactions promoted by the IR procedure in Anx-A1$^{-/-}$ mice. Taken together, these in vivo data strongly suggests that nedocromil exerts its protective effects in the inflamed microcirculation through the anti-inflammatory protein Anx-A1. Haemodynamic parameters were also measured. Administration of nedocromil (2 or 20mg/kg) significantly increased cell flux and wall shear rate in the WT mouse relative to PBS treatment (Table I; see supplementary data), but the compound did not alter the haemodynamic parameters in Anx-A1$^{-/-}$ mice (Table II; see supplementary data).

Measurement of myeloperoxidase (MPO) in the mesenteric tissue

PMN accumulation into the mesenteric tissue was also assessed by quantifying deposition of MPO by infiltrated cells. Mesenteric tissue samples from WT and Anx-A1$^{-/-}$ mice that had been subjected to IR exhibited a significant increase in MPO: 0.26 ± 0.04 U/mg to 1.06 ± 0.08 U/mg and 0.49 ± 0.02 U/mg to 1.58 ± 0.23 U/mg for WT and Anx-A1$^{-/-}$, respectively (Figure 1C). Mesenteric tissue samples from WT mice that had been treated with nedocromil (2mg/kg) had a significantly less MPO activity (0.56 ± 0.06 U/mg) but, importantly the inhibitory effect of nedocromil was lost (1.64 ± 0.19 U/mg) in the Anx-A1$^{-/-}$ mice. Of interest, Anx-A1$^{-/-}$ mouse tissue displayed a trend to higher MPO values than WT tissue samples (Figure 1C).

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Effects of nedocromil on human neutrophil-endothelium interaction

HUVECs, stimulated with TNF-α for 4 h (a time point that allows sufficient time for *de novo* synthesis of adhesion molecules [20]) were used in this assay. Activation of HUVEC monolayers provoked a marked capture of PMNs and both rolling and adherent leukocytes could be visualized and counted off-line. Whilst nedocromil (10nM), pre-incubated for 5 min with the PMNs, had no effect on the number of leukocytes rolling or captured on the endothelium, the compound significantly decreased (>50%) the number of cells adherent on the endothelium (n=3, $p<0.05$; Figure 2A).

To ascertain the role of Anx-A1 in the mechanism of action of nedocromil, the effect of a specific neutralizing anti-Anx-A1 antibody was tested. PMNs were pre-incubated for 20 min with 10 μg/ml neutralizing Anx-A1, or irrelevant isotype-matched, monoclonal antibodies and then incubated for 5 min with nedocromil (10nM). The anti-adhesive effect of nedocromil was almost completely reversed ($p<0.05$) by the anti-Anx-A1 antibody (Figure 2A). The non-neutralizing control monoclonal antibody was without any effects in the system (data not shown).

Since Anx-A1 exerts its anti-inflammatory effects through the activation of formyl peptide receptors, we attested the effect of highly specific blocking anti-FPR1[22] and anti-FPR2[20] antibodies (5μg/ml, 1h prior treatment; Figure 2B). Interestingly, the anti-adhesive effect of nedocromil sodium was partially abrogated by either antibody but the combination of both could totally blocked nedocromil inhibitory adhesive effect of the PMNs on the monolayer. The non-neutralizing monoclonal antibody was without any effects in this system (data not shown).

Effect of nedocromil on FPR family receptors

To provide additional support for our notion that Anx-A1 acts through FPR receptors in this flow chamber system, the expression of FPR-1 and FPR-2 receptors on PMN cell surface were analyzed by FACs after treatment with cromones. Figure 3 indicates that nedocromil rapidly (within 5 min) causes internalisation of both receptors (15-20%). Unrelated anti-inflammatory drugs were assessed as positive controls.

Collectively, these *in vivo* and *in vitro* data support our hypothesis that cromones can exert their anti-PMN effects by releasing endogenous Anx-A1. Thus, the effect of these drugs on Anx-A1 disposition in human PMN was further investigated.

Effect of cromones on Anx-A1 phosphorylation and secretion by human PMNs

To address our next aim, the phosphorylation of PKC and Anx-A1 in cell lysates of PMNs pre-treated with different drugs was determined. Western blotting analysis reveals the rapid phosphorylation of protein kinase C (PKC) in human PMN cell lysates after 5 min treatment with either nedocromil (10nM), cromoglycate (10nM) or ketotifen (10nM) (Figure 4A). This is accompanied by an increase in phosphorylated Anx-A1 at Ser^{27}, which we have shown to be essential for secretion of the protein [13, 16]. No changes in total amount of Anx-A1 could be detected in the PMN extracts (Figure 4A). The same phosphorylation pattern of the

protein was observed when PMNs were treated with either dexamethasone (2nM) or okadaic acid (10nM).

The increments in phospho-Ser[27] Anx-A1 would be indicative of protein mobilization and secretion, according to our model. We next therefore monitored cell-surface Anx-A1 in human PMN, using flow-cytometry analysis. Under basal conditions, very few PMN (<3%) displayed Anx-A1 on their plasma membrane (as detected with a highly specific monoclonal antibody; Figure 5A and B), while pre-treatment with ketotifen (10nM) or dexamethasone (2nM) significantly increased the proportion of positive cells (Figure 5B), with cromoglycate, nedocromil and okadaic acid being somewhat less active in this assay (Figure 5B).

The final step of Anx-A1 mobilization in activated PMN is secretion in the medium. Thus, we next assessed, using a validated ELISA [23], whether we could detect release of Anx-A1 into the extracellular mileu following cromone treatment. Nedocromil, cromoglycate and okadaic acid were found to be more effective inducers of Anx-A1 release into the medium than either ketotifen or dexamethasone (Figure 5C). These observations could explain, in part, the apparent discrepancies observed between the drug effects in the static adhesion assay, presented in Figure 1S (see supplementary data).

Effect of cromones in an acute model of inflammation

Finally, we tested whether the effects of cromones on PMN/endothelium interaction could be translated to attenuation of PMN infiltration in a model of acute peritonitis. The marked (>10 million cells) PMN accumulation promoted by zymosan was susceptible to inhibition by two unrelated anti-inflammatory drugs, the melanocortin analogue melanotan II and indomethacin (a cyclo-oxygenase inhibitor; Figure 6A). As neither of these drugs act through an Anx-A1-dependent pathway, they displayed similar inhibition of cell influx in both WT and Anx-A1$^{-/-}$ mice (MTII: 26% and 33%; indomethacin: 80% and 83%, respectively).

Nedocromil was tested next in this model; probably because of poor pharmacokinetics, the doses required to inhibit PMN migration in this model are higher than one would anticipate from the *in vitro* data, with doses below 10mg/kg being without effect (Figure 6B). Importantly though, as in the case of dexamethasone[24], nedocromil acts in a genotype dependent manner with doses of ranging from 10 to 50 mg/kg, producing a graded inhibition between 5% and 49% in WT mice, whereas no consistent effect was observed in Anx-A1$^{-/-}$ mice. Cromoglycate (data not shown) produced a comparable inhibitory profile although does of 50-200 mg/kg were necessary to inhibit PMN migration in this model.

Discussion

There is now compelling evidence supporting the notion that mast cells regulate leukocyte influx in disease. Indeed efficacy of 'mast cell stabilising drugs' as well as the phenotype of mast cell-deficient mice support this concept in lung inflammation [25], gut inflammation [26], blood-brain barrier permeability [27], plasma extravasation [28] and intestinal inflammation [29]. Despite this, others studies show how leukocyte influx can also occur in a mast cell-

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independent manner [9] and that mast cell stabilisers may act on inflammatory cells other than the mast cell, although a mechanism that could explain such anti-inflammatory effects has never been offered. Our study indicates Anx-A1 as a major molecular target for these effects of the cromones.

We have recently reported that Anx-A1, a GC-regulated anti-inflammatory protein, is secreted by anti-allergic drugs when cells have first been ‘primed’ with low GC doses [16]. We proposed that this occurred secondary to the PKC activation (triggered by GCs) necessary to initiate Anx-A1 phosphorylation and that the cromones potentiated this effect by inhibiting a phosphatase (probably PP2A), which limits PKC activation and activity at the plasma membrane. For this reason, the effect on Anx-A1 phosphorylation and secretion by PMN, of a well-characterised PP2A inhibitor such as okadaic acid was also assessed. Indeed, in line with our previous study using the U937 promonocytic cell line, a correlation between the effects of cromones and okadaic acid on Anx-A1 distribution in the PMN was observed. Thus, the model whereby two processes are required for Anx-A1 secretion, phosphorylation as a pre-requisite for externalization is not limited to the mast cell and monocyte, but also valid for human PMN, hence likely to be a paradigm in the biology of this protein.

We have not addressed the actual mechanism of Anx-A1 secretion from the PMN in this study. These cells contain large amounts of the protein (up to 4% of total intracellular proteins [30]); the intracellular distribution is complex [31] with a high proportion (~50-60%) of Anx-A1 being located in gelatinase granules [32], which are externalised along with their contents following adherence to endothelial cells, after which it is eventually cleaved by PR3 and other proteases [33]. Depending upon the mechanism of PMN activation Anx-A1 may be also released attached to microparticles [34]. It is not clear whether it is the gelatinase pool or the cytosolic (not in granules/vesicles) Anx-A1 pool that is the target for PKC in the human PMN, but in our experiments (Figure 2S, see supplementary data) using markers to identify the different granule subsets in PMN, it seemed the latter pool of Anx-A1 to be mobilised following PKC-mediated phosphorylation.

Since Anx-A1 brings about anti-inflammatory effects through potent inhibition of leukocyte migration process, we hypothesised that the functional release of the protein by cromones could not only explain their inhibition of eicosanoid release [16] but also underlie their inhibitory effects on cell influx in acute and chronic inflammation. Mast cell activation produces a sustained leukocyte influx through release of mediators such as histamine. This can occur in an IgE-dependent, as well as independent, manner. One example of the latter is IR injury in which mast cells have shown to be crucial regulators of leukocyte influx (for review see [7]). Therefore, in this present study, we have used the mouse mesenteric microcirculation inflamed with an IR procedure and monitored by intravital microscopy the process of leukocyte recruitment onto the post-capillary venule endothelium. In line with previous studies conducted in the rat using cromoglycate or ketotifen [35], nedocromil induced a decrease in leukocyte adhesion and emigration. This was absent in our Anx-A1$^{-/-}$ mouse colony providing a strong evidence for an important role for this effector of endogenous anti-inflammatory regulation. Conscious that Anx-A1$^{-/-}$ mice exhibit a tendency to exacerbated inflammatory responses [36, 37], we have also tested a supramaximal dose of

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nedocromil to ensure that its effect was not masked by the more pronounced inflammatory response seen in these knockout mice. It should be noted that no difference between WT and Anx-A1$^{-/-}$ mice could be demonstrated in this experimental settings, indicating that the endogenous protein is not operative within the time frame used in these experiments. In fact, we know that Anx-A1$^{-/-}$ mice display augmented cellular responses in the mesenteries post-IR at later time-points (>6h; unpublished data).

Moreover, using nedocromil as a prototype, we could assess the anti-inflammatory properties of cromones, in a model where an intense PMN accumulation occurs. In this experiment, a significant inhibition and, importantly, a marked reliance on endogenous Anx-A1, was observed. The effect of nedocromil was lost in Anx-A1$^{-/-}$ mice, in line with that reported for dexamethasone: historical data from our laboratory shows that given at 1mg/kg this steroid provokes an acute Anx-A1-dependent inhibition reaching ~80% inhibition in WT, but only 9% inhibition in Anx-A1$^{-/-}$ mice. As expected, the assay can discriminate the contribution of endogenous Anx-A1 since a melanocortin receptor agonist and indomethacin provoked similar degrees of inhibition of PMN accumulation both in WT and Anx-A1$^{-/-}$ mice.

These actions of the cromones can be compared to those obtained with dexamethasone, used as a positive control since it also relies, in part, on Anx-A1 mobilization for its anti-leukocyte effects [37, 38]. Additional evidence that this mechanism is relevant to the anti-inflammatory properties of cromones comes from our experiments using a specific neutralizing anti-Anx-A1 antibody in a flow chamber model. Human PMN-endothelial monolayer interactions were monitored here under defined flow conditions using a human system *in vitro* that physiologically resembles the events occurring in the microcirculation during inflammation. HUVECs were stimulated with TNF-α for 4 h to promote human PMN adherence [39]. While nedocromil was without any effect on the capture and rolling of the PMN onto the HUVECs, a significant decrease in the number of PMN adherent to the monolayers was observed: this inhibition was abolished when the PMN were pre-treated with a specific anti-Anx-A1 neutralising antibody. This suggested that the anti-migratory effect of nedocromil on neutrophil recruitment is an Anx-A1 mediated event.

This study mainly addressed the mechanism by which the cromones exert their inhibitory effect on leukocyte recruitment (and used protocols to focus on the PMN as main target), and we have not investigated their effect on other molecules implicated in the trafficking cascade. It is described in the literature that cromoglycate can down-regulate vascular CD54 (or ICAM-1) expression in a rat lung ischemia-reperfusion model [6], on endothelial cells interacting with PBMC of asthmatic patients [40] or in biopsies of their bronchial mucosa [41]. These effects could contribute to the cromone inhibitory effects seen on leukocyte recruitment in the IR model since increased levels of vascular ICAM-1 are to be. However, in the flow chamber assay, TNF-α-stimulation of HUVECs cells for 4 h increases E-selectin (CD62E) and little CD54 (which expression peaks at 16h of TNF-α stimulation; not shown). Moreover, it is unlikely to be working via any targets on the endothelium, since the drugs were added to the neutrophils prior to the flow assay.

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In conclusion, we have established that cromones can act directly upon human and mouse PMN, to regulate their recruitment on activated endothelial monolayers or post-capillary venule endothelium, respectively. We also provide the first evidence, in vivo as well as in vitro, that these effects require the endogenous anti-inflammatory protein Anx-A1, showing – in human PMN – externalization and release of this protein. These findings could explain some observations originally made by Altounyan (reviewed in [42]) who speculated that cromones might be endowed with clinical properties similar to corticosteroids. In addition to elucidating a mechanism through which cromones exert anti-inflammatory properties, our study also suggests further uses for these drugs in the context of pathologies driven by excessive PMN activation examples being vasculitis and gouty arthritis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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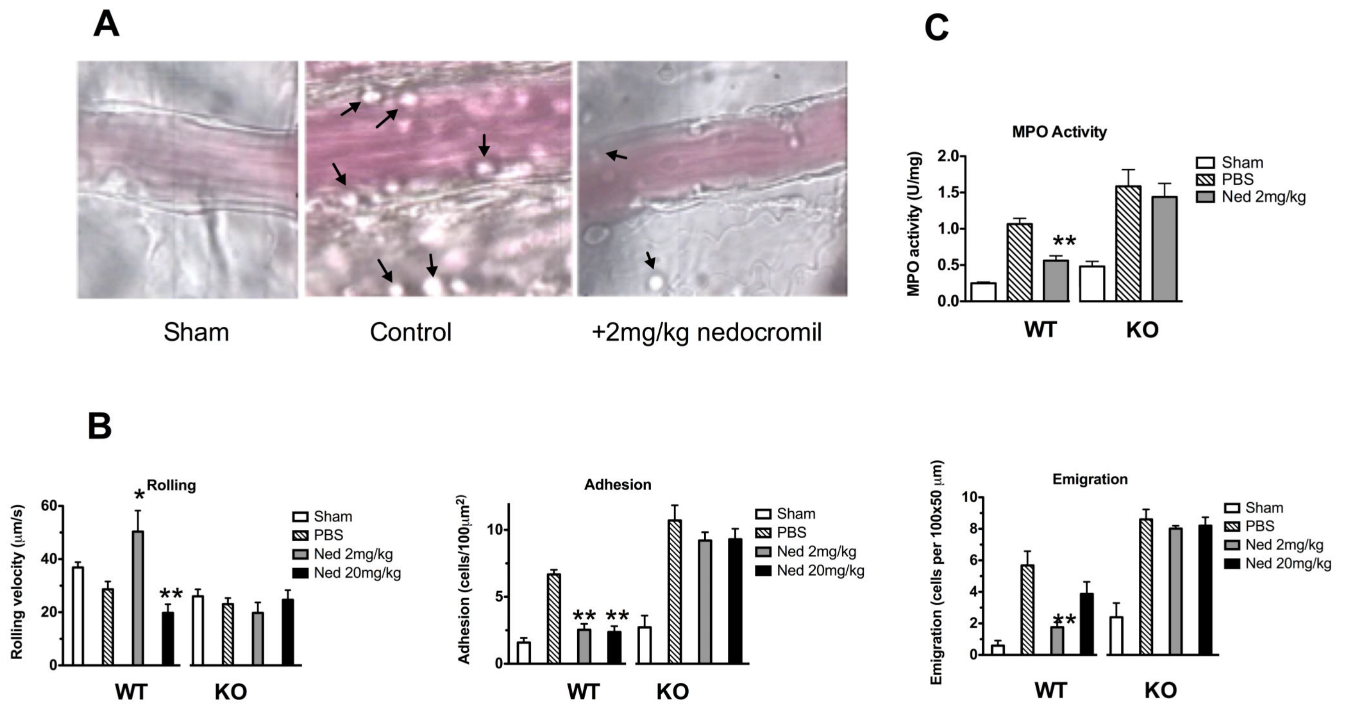

Figure 1. Nedocromil inhibits PMN migration and MPO tissue levels in a mouse model of ischaemia-reperfusion injury

(A) Images are representative pictures of the mouse mesenteric microcirculation as observed in sham-operated mice (left hand frame) and following occlusion and reopening of the SMA (centre frame) and after administration of nedocromil (right hand frame). (B) Inhibitory effect of nedocromil on the leukocyte-endothelium interaction in IR inflamed mesenteric vessels of WT mice is abrogated in Anx-A1 KO mice. (C) Myeloperoxidase (MPO) releases in injured mesenteric tissues following IR injury. Data are mean ± SEM of n=6 mice per group. $^{**}p<0.01$ vs sham group, $^{*}p<0.05$ vs PBS group (see the Supplemental Materials file for details).

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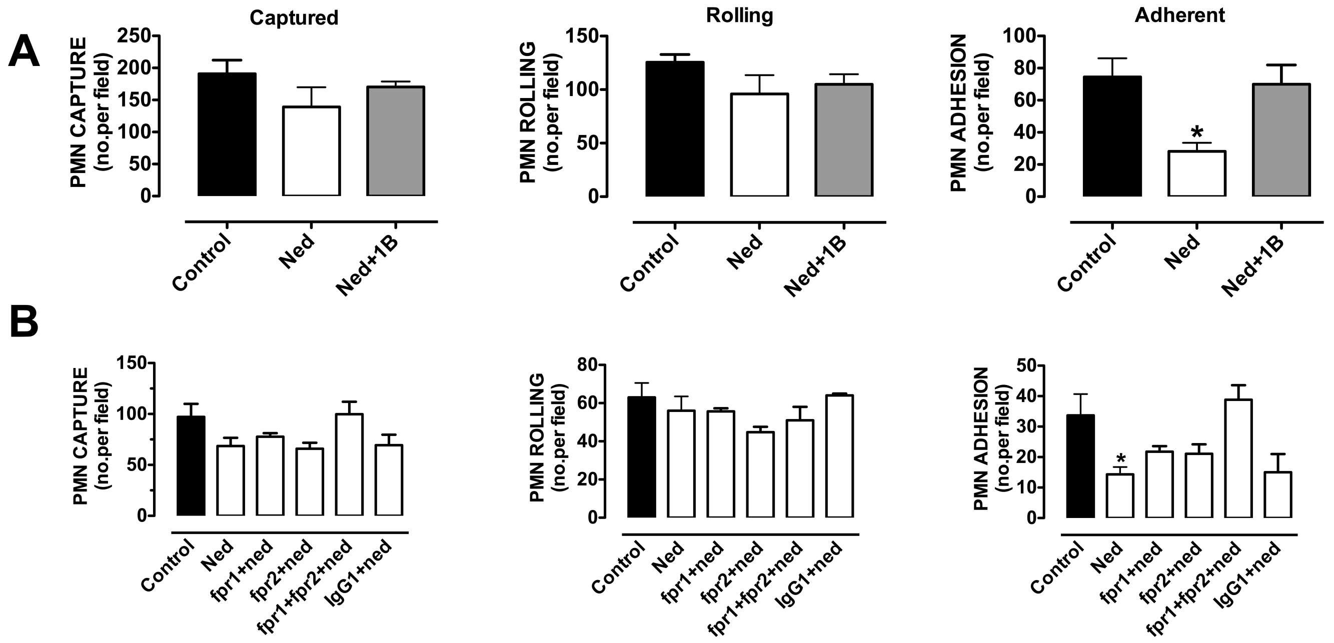

Figure 2. Cromones inhibit PMN adhesion to HUVEC

Inhibitory effect of nedocromil on PMN interaction with HUVECs under flow is abrogated (A) by the use of a specific neutralizing anti-Anx-A1 antibody (20μg/ml) and (B) by the use of a combination of specific blocking anti-FPR1 and anti-FPR2 antibodies (5μg/ml). Data are presented as mean ± SEM of 3 independent experiments, $^{*}p<0.05$ vs control group (see the Supplemental Materials file for details).

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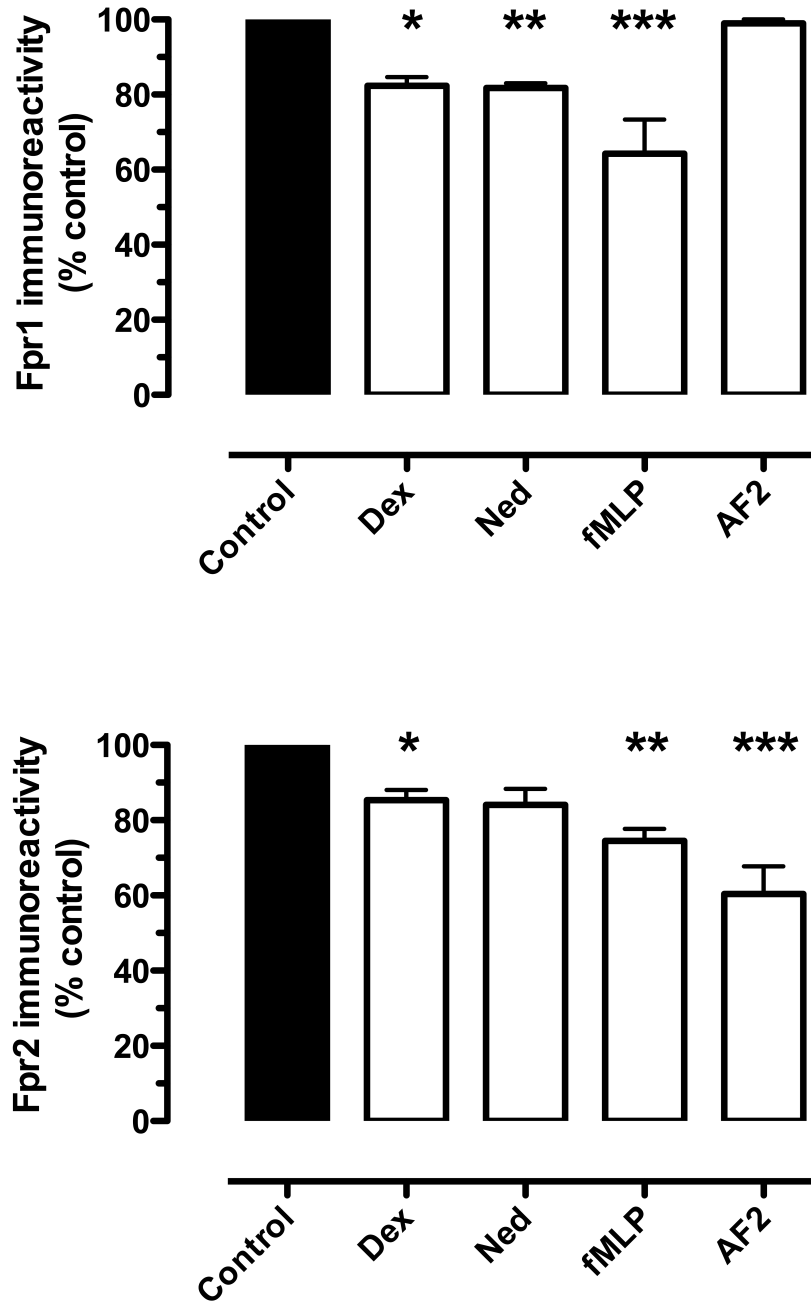

Figure 3. Assessment by FACS analysis of FPR-1 and FPR-2 receptor internalization in PMNs produced by cromones

Human PMN were incubated with either nedocromil (10nM) or dexamethasone (2nM) for 5min at 37°C; after immediate transfer to 4°C, FPR1 and FPR2 cell surface expressions were determined with specific antibodies and flow cytometry analysis. Data are presented as mean ± SEM of 3 independent experiments, $^{*}p<0.05$ vs control group (see the Supplemental Materials file for details).

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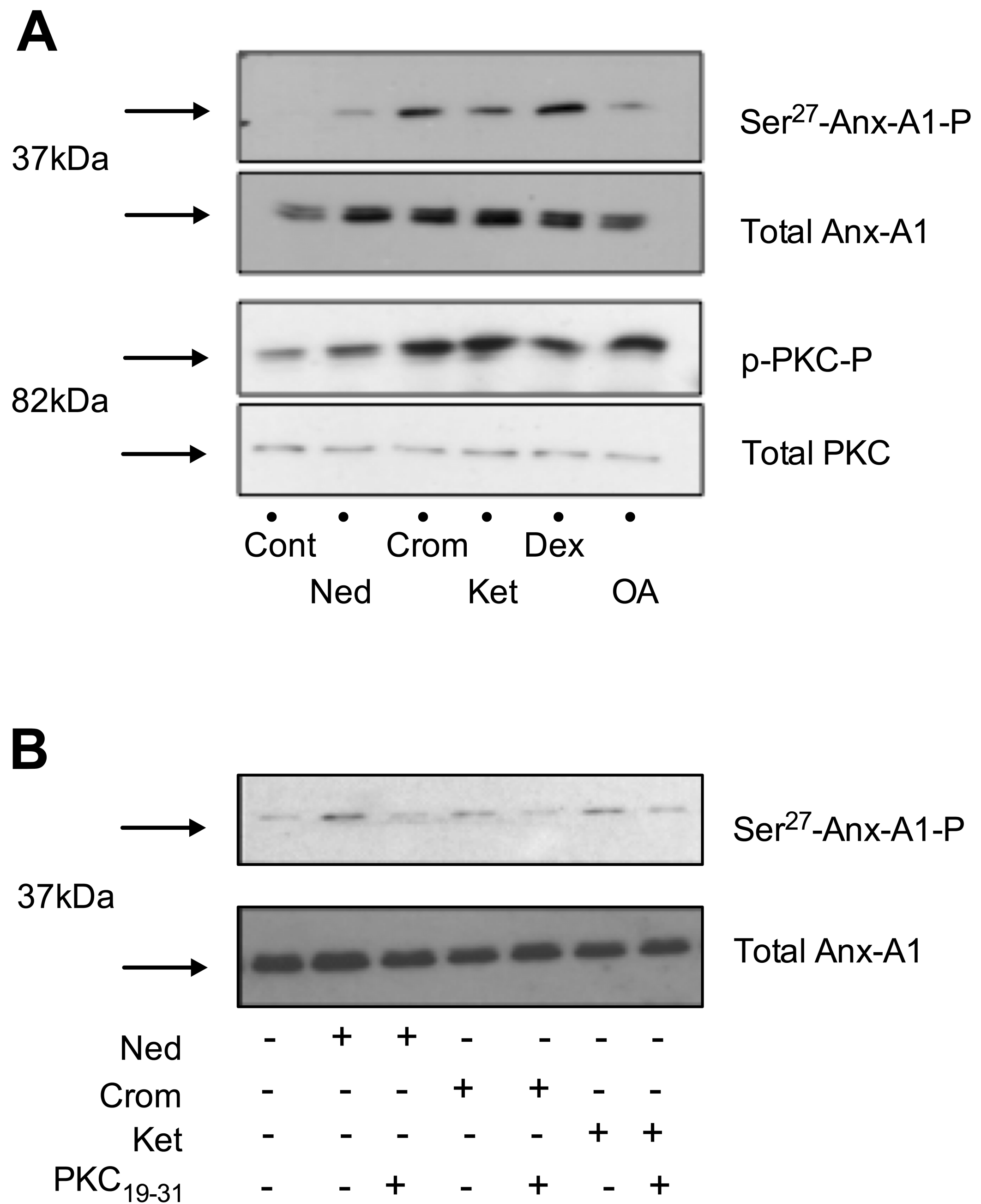

Figure 4. Crucial Anx-A1 phosphorylation by human PMN following treatment with cromones

(A) Effect of the mast cell stabilisers on Anx-A1 and PKC phosphorylation. (B) Suppressive effect of $PKC_{19\text{-}31}$ peptide on Anx-A1 phosphorylation (see the Supplemental Materials file for details).

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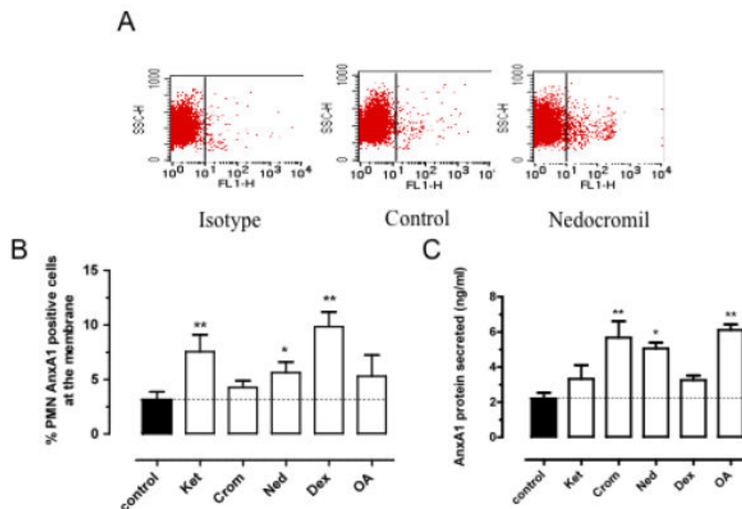

Figure 5. Anx-A1 membrane expression and secretion by FACs and ELISA

(A) Representative dot plot of membrane expression of the Anx-A1 positive cell population amongst resting (control) or treated isolated human PMN. (B) Represents the % of positive PMN expressing Anx-A1 on cell surface after cromone treatment. (C) ELISA assay measuring Anx-A1 protein in the supernatant of PMNs. Data are presented as mean ± SEM of 3 independent experiments, $^{**}p<0.01$ or $^{*}p<0.05$ *vs* control (see the Supplemental Materials file for details).

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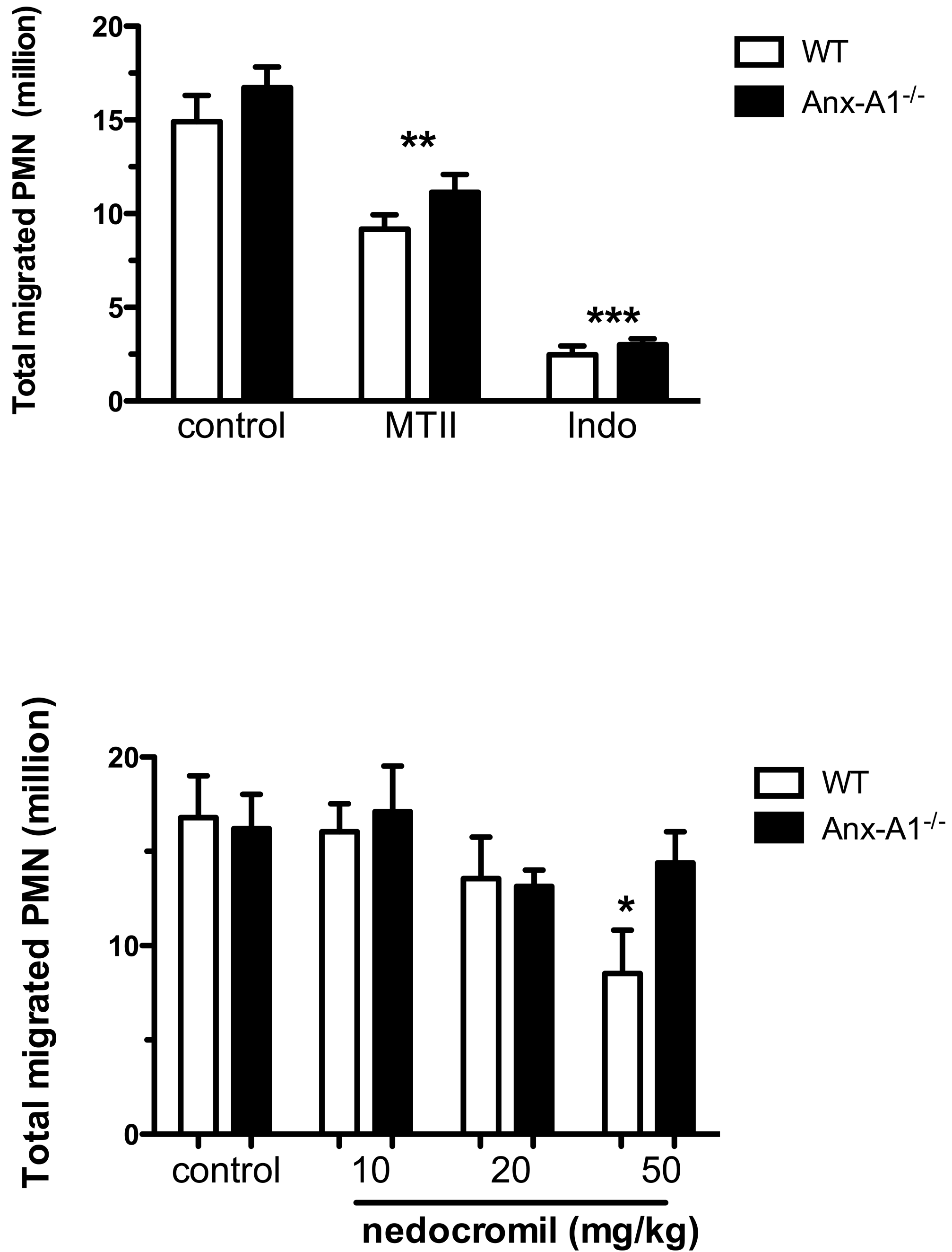

Figure 6. Cromones inhibit the intense PMN accumulation of the zymosan-induced peritonitis model

(A) Effect of unrelated anti-inflammatory drugs on the number of PMN migrated. (B) Effect of increasing doses of nedocromil on PMN migration into the inflamed peritoneal cavity. Data are presented as mean ± SEM of n=6 mice per group,*p<0.05 *vs.* control group (see the Supplemental Materials file for details).