



Review

Anti-allergic drugs and the Annexin-A1 system

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Abstract:

This paper, which was presented at the 17th JMRC ‘John Robert Vane Memorial’ Symposium, describes some recent work from the authors’ laboratory that provides a tentative explanation for the anti-inflammatory effects produced by the cromoglycate-like anti-allergic drugs. Some of the implications of this finding are discussed.

Key words:

sodium cromoglycate, sodium nedocromil, glucocorticoids

Introduction

With the exception of the new ‘biological’ anti-inflammatory agents such as the anti-TNF agents, most drugs we use to treat inflammatory diseases entered into our therapeutic repertoire a great many years ago, although an understanding of the mechanism of their action usually came much later. For example, aspirin was first synthesized in the 1890s but its mechanism of action was not understood until the work of John Vane and his colleagues in the 1970s [19]. Paracetamol (acetaminophen) is even older and still poses mechanistic problems today. Glucocorticoids are another case in point. Although they were introduced into clinical medicine in the 1950s, we are still grappling with the complexities of their action and learning to exploit that knowledge in order to design superior drugs.

It is sobering to reflect on the fact that ignorance of the mechanistic basis of the action of such drugs has neither prevented their effective use in the clinic nor has it noticeably impaired progress in finding new anti-inflammatory agents with similar mechanisms. Many highly effective non-steroidal anti-inflammatory drugs (e.g., indomethacin, phenylbutazone) were discovered and marketed prior to the elucidation of aspirin’s mechanism of action and many potent glucocorticoid analogues (e.g., dexamethasone) were synthesized long before we had a rudimentary grasp of their mechanism of action.

All of the above comments also apply to the ‘cromoglycate-like’ or ‘cromone’ anti-allergic drugs. Di-sodium cromoglycate was discovered in the late 1950s [5] and introduced as a treatment for allergic asthma. Other similar drugs were subsequently introduced (e.g., nedocromil [3]) and the therapeutic indications for these drugs (often referred to as ‘cromones’)

were extended to cover other allergic conditions (e.g., allergic conjunctivitis, intestinal allergies). While not as potent as the glucocorticoids, these drugs have retained a niche role in the treatment of asthma and allergies and have a reputation for being very well tolerated.

Early in their history, the cromoglycate-like drugs were dubbed ‘mast cell stabilizers’ on the basis of the fact that they were able to prevent the release of mast cell histamine when stimulated by various agonists. However, little else was known about their mechanism of action. My colleagues and I have now made a series of observations that shed some light on this mystery. Interestingly, there seems to be a link between the action of these drugs and the properties of a protein already characterized by us as a mediator of glucocorticoid action, Annexin-A1 (Anx-A1). We will therefore begin this paper by discussing some pertinent aspects of Anx-A1 biology; for a recent complete review of the next section the reader is referred to [6].

A brief summary of Anx-A1 in inflammation

Anx-A1 is a 36 kDa monomeric protein that is found in many differentiated cells, particularly those of the myeloid lineage. We now know it to be a member of a superfamily of proteins that are found in most eukaryotic cells and are of ancient evolutionary origin. Structurally, these annexins comprise a number of homologous repeated core domains that primarily bind calcium and negatively charged phospholipids. These domains are attached to an N-terminus of variable length. There are 12 annexins in mammals and Anx-A1 (so numbered because it was the first to be cloned) has four repeating units in the core domain. These units can bind calcium and, under appropriate circumstances, phosphatidylserine and other negatively charged phospholipids.

Prior to its cloning, sequencing and characterization, Anx-A1 (also known as ‘macrocortin’, ‘renocortin’, ‘lipomodulin’ and ‘lipocortin’ at various times) was recognized through its biological activity. It was detected in the conditioned media or perfusate of tissues or cells that had been treated with glucocorticoids and was surprisingly found to mimic the action of these drugs in a number of *in vitro* and *in vivo* systems. The synthesis and release of this factor were hypothesized

to account for some of the anti-inflammatory actions of these agents.

These early findings and suspicions were fully confirmed by later work that tested this hypothesis using the highly purified recombinant protein, Anx-A1-deficient transgenic animals, neutralizing antibodies and anti-sense agents. Anx-A1 is now known to play a key role in several important host defense responses including inflammatory resolution and T-cell signaling.

The mechanism by which glucocorticoids and other factors control Anx-A1 has also been investigated in some depth. Surprisingly, in many cells of the innate immune system such as macrophages glucocorticoids have been found to have a dual action on Anx-A1 disposition, promoting not only its synthesis through genomic action but also the release of pre-existing pools of Anx-A1 from the cytoplasm of these cells. This latter mechanism is triggered by the PKC-mediated phosphorylation of Anx-A1 on Ser²⁷ and other residues followed by the translocation of Anx-A1 to the plasma membrane and its release from the cell [15].

Once released Anx-A1 is able to interact with its target cells in an autocrine or paracrine manner utilizing receptors of the FPR family, probably ALX/FPRL1 in man and its homologue Fpr2 in the mouse. Downstream signaling events mediated through this receptor culminate in the generally inhibitory effect that Anx-A1 has on cell activation [8].

Proteolysis of Anx-A1 is crucial in determining the duration of action of the protein and may be a factor in some pathologies such as Wegener’s granulomatosis where excessive activation of the PMN can be traced to inappropriate cleavage of Anx-A1 by the PR3 protease.

Anx-A1 and mast cells

Mast cells, one of the main targets of cromoglycate action, also contain abundant Anx-A1 and rapidly respond to glucocorticoid stimulation with an increase in Anx-A1 mRNA [12]. These cells also express the appropriate Anx-A1 receptor (FPRL1 [1]). The subcutaneous injection of the mast cell secretagogue 48/80 into the flank of Anx-A1 null mice produces a greater wheal than that in wild type animals (see Fig. 1A). In addition, mast cells in Anx-A1 null mice

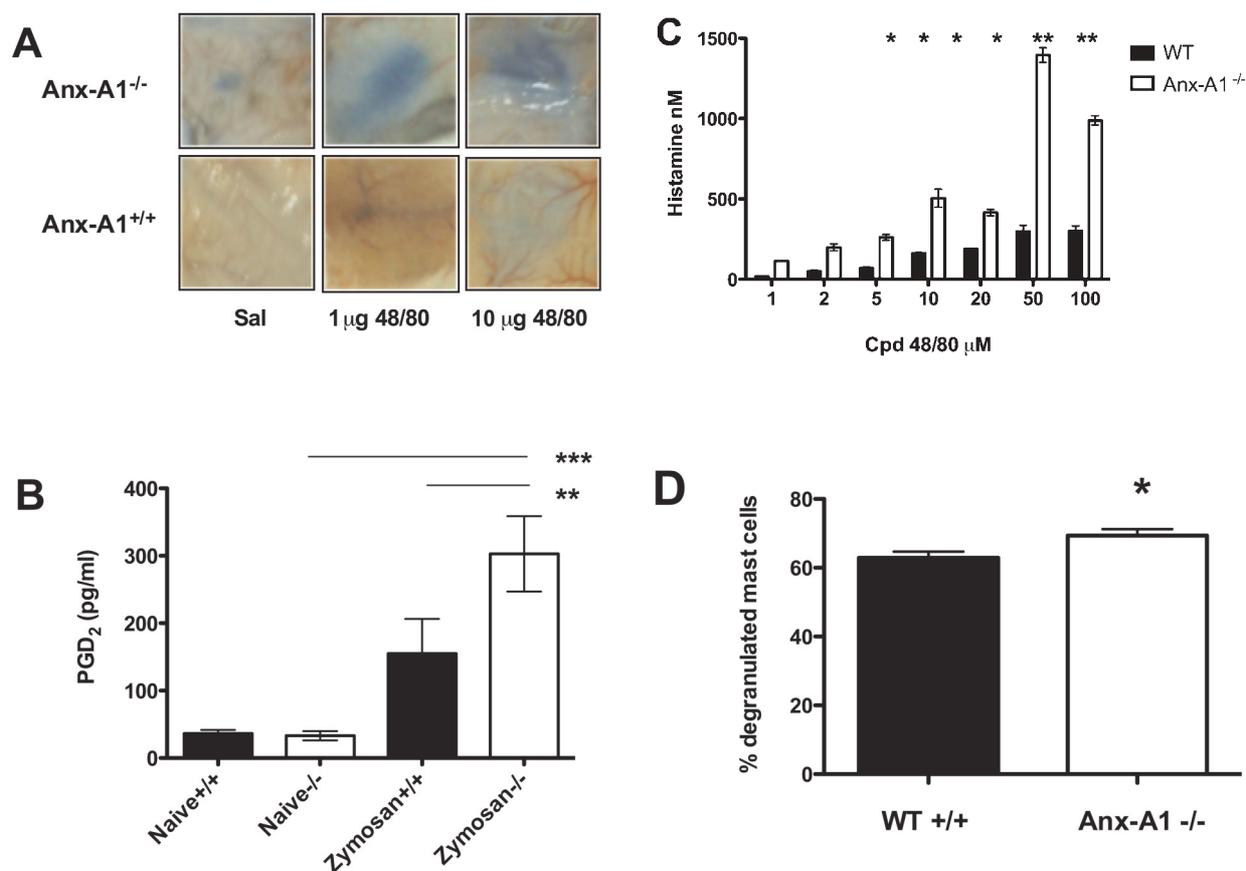


Fig. 1. Anx-A1 null mice are more sensitive to mast cell degranulation provoked by inflammatory stimuli. Panel **A**: Extravasation of Evan's blue dye following a subdermal injection of 0.1 ml saline with 1 µg or 10 µg compound 48/80 in the mouse. The Anx-A1^{-/-} mice respond strongly to this stimulus but these doses have little effect in Anx-A1^{+/+} mice (Ayoub et al. unpublished 2009). Panel **B**: Enhanced release of PGD₂ from mast cells in the peritoneal cavity of Anx-A1^{-/-} mice *in vivo* following the induction of zymosan peritonitis compared to the Anx-A1^{+/+} controls. PGD₂ is a specific marker of mast cell activation (Ayoub et al. unpublished 2009). Panel **C**: Release of histamine from mast cells by escalating concentrations of compound 48/80 *in vitro* is enhanced in cells taken from Anx-A1^{-/-} mice. The preparation used was mixed peritoneal lavage cells. There was no difference in the numbers of mast cells between the phenotypes. Histamine was estimated by ELISA (Dufton et al. unpublished 2009). Panel **D**: The percentage of spontaneously degranulated mast cells in peritoneal lavage cells taken from wild type compared with Anx-A1^{-/-} mice. A small but significant difference is consistently found between the two phenotypes (Ayoub et al. unpublished 2009). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ when compared to appropriate controls using ANOVA

are more prone to degranulation and release more PGD₂ and histamine than wild type cells in response to various challenges (see [7] and Fig. 1B, C and D). All these observations suggest that under normal circumstances Anx-A1 exerts a tonic inhibitory influence on mast cell reactivity.

Congruent with this notion is the observation that Anx-A1 is also an active inhibitor of mast cell activation in a therapeutic context; administration of the Anx-A1 N-terminal peptide Ac 2-26 to rats strongly inhibits antigen-induced release of histamine in the pleural cavity [1].

The anti-allergic cromones

The prototypical drug in this group, disodium cromoglycate, was introduced into clinical medicine in the early 1960s. It arose from a Fisons program aimed at studying the anti-spasmodic properties of an Egyptian herb called Khellin. The events leading to the discovery of this compound, involving considerable self-experimentation by Roger Altounyan, have become part of pharmacological legend and will not be reviewed here (see [9]). Disodium cromoglycate was originally introduced for the treatment of mild-

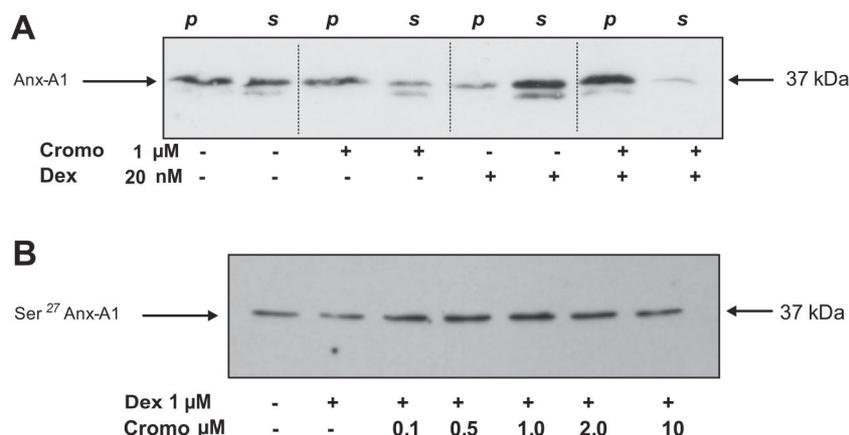


Fig. 2. The effect of co-administration of a glucocorticoid (dexamethasone) and sodium cromoglycate on the location and quantity of Ser²⁷ phospho-Anx-A1 in U937 cells. Panel **A**: U937 cells (differentiated the day prior with PMA) were treated with various combinations of the glucocorticoid dexamethasone (20 nM) and sodium cromoglycate (1 mM) for 5 min after which the subcellular location of Ser²⁷ Anx-A1 was determined by Western blotting. Cromoglycate itself has little effect on the amount of Ser²⁷ Anx-A1 in the cells. Dexamethasone strongly stimulates the phosphorylation of the protein but this phosphorylated protein remains mainly in the cytosolic fraction. In the presence of cromoglycate, however, the phosphoprotein is translocated into the (13,000 × g pellet) membranous fraction of the cells (Vo et al. unpublished 2009). Panel **B**: The accumulation of Ser²⁷ Anx-A1 in U937 cells triggered by dexamethasone after 5 min treatment is strongly enhanced in the presence of escalating concentrations of cromoglycate (Yazid et al. unpublished 2009)

moderate asthma and subsequently incorporated into other anti-allergic medications such as eye-drops. It remains a feature of the allergist's arsenal today and has established a reputation as a moderately potent and very safe drug.

Nedocromil was developed (also by Fisons) in the late 1970s [3]. It is chemically closely related to cromoglycate and shows comparable or even greater efficacy in the clinical setting. Other drugs have been developed that apparently act in a similar way, including a number of H₁ antagonists that appear to possess additional anti-inflammatory effects above and beyond those that might be expected *via* the antagonism of histamine; these drugs appear to have a 'cromoglycate-like' action on mast cells by preventing them from degranulating and releasing histamine [4].

Initial studies on the mechanism of action of cromoglycate and nedocromil suggested that they blocked mast cell degranulation triggered by various stimuli and thus these drugs became known as 'mast cell stabilizers' [5]. In fact, many other anti-inflammatory actions of cromoglycate-like drugs that have been reported include inhibition of leukocyte activation [10] and inhibition of eicosanoid [11] and cytokine [13] release from different cell types. It is now widely believed that the anti-asthma effects of cromoglycate and nedocromil are due to these anti-inflammatory actions (e.g., [2]).

Investigations into the mechanism of action of cromoglycate and other cromones in mast cell degranulation have led to several anomalous observations. For example, the timing of the administration of the drug in relation to the antigen or other challenge is crucial. If the drug is given too long before the challenge then its blocking effect is greatly diminished. Strong tachyphylaxis has been observed in that the blocking effect of the drug cannot be repeated within a defined period (e.g., [18]). Observations such as this led some early commentators to speculate that cromoglycate caused the release of a factor that once depleted required a period of time to replenish before the next challenge could be effective. Other anomalies observed with these drugs include the finding that the concentration required for inhibition of mast cell degranulation varies considerably depending upon the source of the mast cell (e.g., [10]).

Several attempts have been made to identify the molecular mechanism through which cromones block mast cell histamine release. A consistent finding has been that the presence of the drug leads to an increase in the presence of some phosphoproteins in the cell, particularly a 78 kDa protein [17], although exactly how this increase could be linked to control of histamine release has not been clear. Some authors have proposed PKC (e.g., [14]) to be important to the mechanism through which these drugs operate, suggesting that they probably inhibit the action of the kinase – al-

though once again, the relevance of this connection to the pharmacological action of the cromones is not clear.

The effect of cromoglycate-like drugs on Anx-A1 export and action

Bearing in mind that the cromoglycate-like drugs act in a manner suggestive of the release of a mediator and that Anx-A1 is clearly implicated in mast cell biology, we formulated the hypothesis that Anx-A1 is released by the cromoglycate-like drugs and that this protein in fact mediates the action of the cromoglycate-like drugs. To investigate the possible mechanisms we set up a very simple model system for studying the biology of Anx-A1 release under closely defined conditions.

The U937 human monocytic cell line offers some advantages in this respect in that these cells are easy to maintain, are responsive to glucocorticoids when differentiated, contain abundant Anx-A1 and produce a convenient biochemical readout of activation: thromboxane (Tx) B₂ production [16], which is suppressed when Anx-A1 is released.

As mentioned above, phosphorylation of Anx-A1 must occur prior to export and PKC had been identified in several studies as the kinase most likely responsible for this action. How the liganded glucocorticoid receptor triggers PKC activation is not yet clear, although in some cells PIP3 kinase has been implicated in this activation.

Early studies from our laboratory suggested some interaction between the action of cromoglycate and the effect of dexamethasone, although the reason for this interaction was unclear at the time. Fig. 2A shows an analysis of the disposition of Anx-A1 in U937 cells revealing the glucocorticoid dexamethasone to produce a small increase in immunoreactive cytosolic Anx-A1; the addition of cromoglycate alone was without effect. However, when the two drugs were combined a dramatic change took place with Anx-A1 being translocated to the membrane fraction of the cells (Vo et al., unpublished).

Yazid et al. [20] studied the dynamics of the phosphorylation of Anx-A1 on Ser²⁷ in response to several glucocorticoids and found the effect to be very rapid with a maximum activation of PKC α/β and Anx-A1 phosphorylation occurring within 5 min. The cromoglycate drugs themselves had little effect on either pathway but surprisingly greatly potentiated the effect of the glucocorticoids. This finding was mirrored by the redistribution of GFP-tagged Anx-A1 in U937 cells (Fig. 3A), by the extracellular accumulation of the protein in response to the two drugs (Fig. 3B) and, dramatically, by the inhibition of TxB₂ generation observed. For example, at 5 min the inhibition of TxB₂ release with 2 nM dexamethasone alone was only < 20% but this inhibition was increased to almost 100% in the presence of 20 nM nedocromil (Yazid et al. [20]). The effects of cromoglycate were similar.

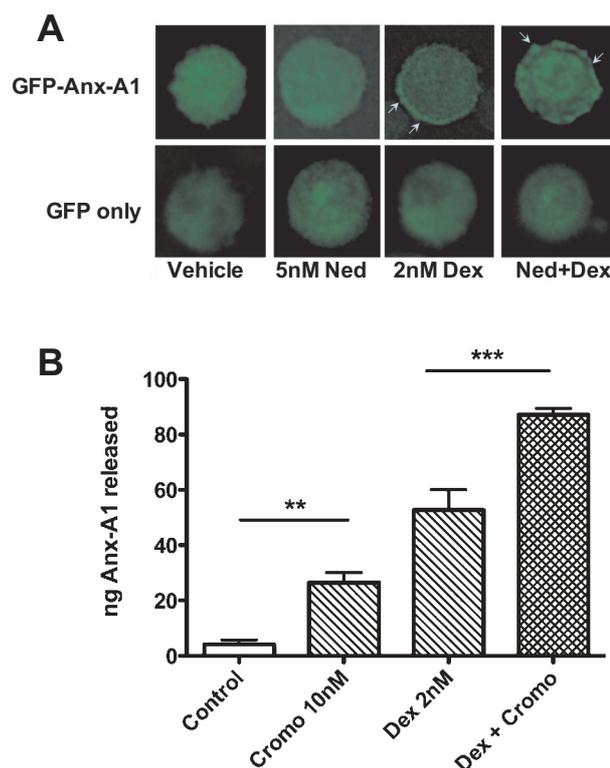


Fig. 3. The effect of glucocorticoid-nedocromil combinations on Anx-A1 intracellular disposition and release from U937 cells. Panel **A**: U937 cells were transfected with an Anx-A1-GFP construct (upper row) or a GFP control construct (lower row). In vehicle-treated cells Anx-A1 is distributed throughout the cytoplasm. The addition of nedocromil has no observable effect; however, dexamethasone produces a noticeable margination of the GFP-labeled protein and this margination is enhanced in the presence of nedocromil (Yazid et al. unpublished 2009). Panel **B**: The release of Anx-A1 into the medium of U937 cells (as estimated using ELISA) following treatment with cromoglycate alone, dexamethasone alone or a combination. Once again the addition of cromoglycate further increases the release of the protein produced by dexamethasone. (Data from Yazid et al. [20], redrawn). ** $p < 0.01$; *** $p < 0.001$ when compared to appropriate controls using ANOVA

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But how did this effect come about? There are several potential ways in which PKC is regulated. Once

activated, the activity of PKC is terminated by phosphatases including PP2A. Additionally, diacylglycerol, the endogenous activator of PKC is rapidly destroyed by diacylglycerol kinases that terminate PKC activation. It therefore follows that inhibitors of either of these two processes should greatly potentiate the dexamethasone-induced increase in Anx-A1 phosphorylation and thus promotes its release from cells. Was this how the cromones were working?

Presumptive evidence of the importance of this mechanism came from an experiment with the PP2A inhibitor okadaic acid. Treatment of cells with this agent greatly potentiated the effect of dexamethasone on Anx-A1 externalization and eicosanoid synthesis. To test this concept more rigorously Yazid et al. [20] tested nedocromil and cromoglycate for the endogenous phosphatase activity present in U937 cell membranes after treatment with dexamethasone and found this activity to be strongly inhibited by these drugs. Inhibition of catalytic activity was also observed when highly purified recombinant PP2A was used as a target enzyme.

Yazid et al. also tested the notion that the enhanced release of Anx-A1 explained the inhibitory action of nedocromil-dexamethasone combination by using an anti-Anx-A1 neutralizing monoclonal antibody. In this case the extracellular addition of this reagent completely reversed the blocking action of the drugs.

Could Anx-A1 release completely explain cromolyn action?

While these studies convincingly demonstrate that the effect of the cromoglycate-like drugs on eicosanoid release in U937 cells is dependent upon the enhanced release of Anx-A1, this finding is obviously not the same as inhibition of mast cell degranulation by these drugs. Could the latter effect be dependent upon enhanced Anx-A1 secretion? Certainly, as discussed above Anx-A1 appears to have a tonic inhibitory role on mast cell activity *in vivo* and the Anx-A1 peptide is a potent inhibitor of mast cell activation. In theory at least, such a mechanism could thus explain the acute effects of cromones on mediator release from these cells.

However, enhanced activation of PKC activity would be expected to have more far-reaching conse-

quences within the cell, such as manifesting an increase in phosphorylation of other proteins that might be of relevance to histamine secretion or other mast cell functions. Interestingly, some likely targets have already been identified. Theoharides [17] identified four phosphoproteins that appeared in mast cells following treatment with cromoglycate at 78, 68, 59 and 42 kDa. The appearance of the 78 kDa protein coincided with the end of the secretory phase triggered by 48/80 and the authors speculated that this protein may be a molecular switch that 'turns off' the secretory process. This protein was cloned and subsequently characterized as the cellular cytoskeletal protein moesin. The authors speculated that differential regulation of this protein at different phosphorylation sites regulated the histamine secretory mechanism. Interestingly, PKC was considered to be the most likely kinase involved in this phosphorylation event.

PKC has also been implicated in cromoglycate action by other researchers [14] in a variety of different models; while here the majority found inhibition of PKC by the drug, differing time courses and methodologies obscure clear interpretation of the issue. Only future studies will clarify the role of this enzyme in the transduction of the manifold effects of the cromoglycate drugs on these cells.

Summary and note on John Vane

Commenting on the role of the pharmacologist, Vane was fond of quoting Gaddum who once stated that one of the roles of the pharmacologist was 'to find out how drugs work'. At the time of Gaddum's article little was known about the mechanism of action of most of our major drugs. During the last half-century things have obviously improved enormously but Gaddum's phrase summarizes what is still a very relevant task for this discipline and there is much work still to be done in this field. John Vane himself was an excellent example of the role of the pharmacologist in this area. His definitive work on the mechanism of action of aspirin and allied drugs was a classic example of how an entire field can be clarified through an insightful observation into drug action. It is very fitting therefore that this symposium be named in his honor.

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