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University of Alberta

Mechanisms of Human Eosinophil Activation

by

Andreas Schwingshackl

A thesis submitted to the faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Physiology

Edmonton, Alberta

Spring 2002

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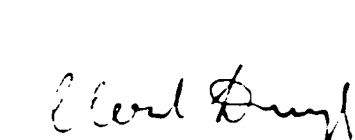

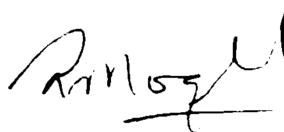

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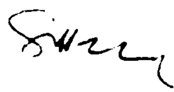

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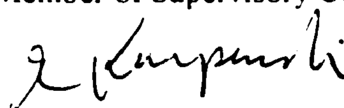

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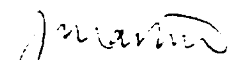

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Preface

This thesis was written in paper format according to the guidelines of the University of Alberta "Thesis Handbook". Each chapter stands alone as a separate document and is written in the style of the Journal of Allergy and Clinical Immunology. All the data in this thesis were generated by the author of this thesis, Andreas Schwingshackl.

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ABSTRACT

Eosinophils are traditionally thought of as protective cells in helminthic infections, but increasing evidence proposes a key role for this cell in allergic diseases including asthma. Among the proinflammatory mediators released by the eosinophil are cationic proteins, cytokines, chemokines and growth factors as well as matrix metalloproteinases (MMPs), lipid mediators and reactive oxygen species. This study focused on two of these products, namely MMPs and superoxide (O_2^-), the first being associated with tissue remodeling and so-called "repair", while the other is tissue damaging. Both of these processes are significant pathophysiological events in obstructive airway disease. We showed that tumor necrosis factor-α (TNF-α) was a potent activator of eosinophils and led to enhanced production and secretion of MMP-9. This increase was regulated at both transcriptional and translational levels. Gene and protein expression of tissue inhibitors of MMPs (TIMPs) varied significantly among patients.

Eosinophil O_2^- is generated following NADPH oxidase activation resulting in respiratory burst. During this process, activation of H^+ channels is thought to provide a mechanism for H^+ extrusion and charge compensation. We found that stimulation of respiratory burst using phorbol 12-myristate 13-acetate (PMA) could be inhibited by several ion channel blockers, especially the Cl^- channel blocker 4,4-diisothio-cyanostilbene-2,2'-disulfonic acid (DIDS). We conclude that activation of Cl^- channels, possibly ClC-3 channels, may constitute an additional pathway for charge compensation during O_2^- generation.

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Although plasma membrane ion channels are thought to be important in stimulus-secretion coupling, little is known about their regulation in eosinophils. We found that eosinophil K^+ but not Cl^- channels were activated by nitric oxide (NO), a molecule found in abundance in inflamed tissues. Although eosinophils expressed mRNA for various K^+ channels, only ATP-dependent K^+ channels were activated by NO. Opening of these channels may help to prevent eosinophil activation in an inflammatory environment.

The results of this study show that under conditions likely to be encountered *in vivo* human eosinophils can be activated and release significant amounts of inflammatory mediators. These processes may involve the activation of different families of ion channels. Selective ion currents across the plasma membrane may ultimately determine the activation status of the eosinophil.

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I wish to acknowledge my parents for their continuous support throughout my education, my two supervisors Drs. Marek Duszyk and Redwan Moqbel for being great mentors throughout the last four years, my committee members Drs. Edward Karpinski and Paul Man for their help and input in my studies, and all my friends in the Pulmonary Research Group.

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List of the K^+ channels tested, the positions of the primers relative to the published data sequences, the expected size of the RT-PCR products, and the base sequence of the primers in the $5' \rightarrow 3'$ direction.

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List of Abbreviations:

1-EBIO	1-ethyl-2-benzimidazoline
4-AP	4-aminopyridine
8-Br-cGMP	8-bromoguanosine 3',5'-cyclic monophosphate
BSA	bovine serum albumin
DIDS	4,4-diisothiocyanostilbene-2,2'-disulfonic acid
DNDS	4,4-dinitrostilbene-2,2'-disulfonic acid
DPC	diphenylamine-2-carboxylate
EDTA	ethylene-diaminetetra-acetic acid disodium salt
EDTA	ethylelediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether) N'N'N'N'-tetraacetic acid
FCS	fetal calf serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSNO	S-nitroso-glutathione
L-NAME	N-nitro-L-arginine methyl ester
MMP	matrix metalloproteinase
NFκB	nuclear factor κB
ODQ	1,2,4-oxadiazole-4,3-quinoxalin-1
PK	protein kinase
PMA	phorbol-12-myristate-13-acetate
PMSF	polymethylsulfonyl fluoride
SDS	soduim dodecyl sulfate
sGC	soluble guanylyl cyclase
SNAP	S-nitroso-N-acetyl penicillamine
SOD	superoxide dismutase
TAME	tosyl-L-arginine methyl ester hydrochloride
TIMP	tissue inhibitor of metalloproteinase
TNF-α	tumor necrosis factor-alpha

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CHAPTER I

General Introduction

1. Historical Background:

Although Warton-Jones in 1846 was the first to observe eosinophils in peripheral blood [1], it was Paul Ehrlich who introduced the term "eosinophile" in 1879 and established methods for ready identification of these cells [2]. He observed that certain peripheral blood cells had numerous intracytoplasmic granules with an affinity for acidic dyes such as eosin. He, therefore, named this cell "eosinophil". In the same year, Ehrlich found that cells with eosinophilic granules were particularly abundant in the bone marrow of patients suffering from leukemia. Subsequently his staining technique was widely used for examination of peripheral blood smears in clinical settings. In the following years and decades, an association of eosinophils and helminthic disease, allergy, asthma and certain cutaneous and malignant diseases was established [3,4]. In the 1960's and 1970's researchers proposed an ameliorating effect of eosinophils on allergic disease, since this cell was shown to degrade mast cell-derived mediators of anaphylaxis [5]. In the late 1970's, the observation that eosinophils or their granule-containing proteins were toxic for helminthic larvae led to the current, widely accepted belief that the teleological function of eosinophils lies in host defense against worms [6]. In the 1980's, scientists reached the consensus that eosinophils, in addition to be protective against parasitic infections, are important pro-inflammatory cells in allergy and asthma. This idea was inspired by findings demonstrating a toxic effect of eosinophil-derived mediators on bronchial epithelial cells [7-10]. In recent years, a rebirth of interest in the eosinophil has occurred, stimulated to a large extent by the possibility of modulating eosinophil function as an effective therapy for asthma and allergic disease. Recent studies indicate that eosinophils may have a more complex effector role than previously appreciated. The cell has been implicated in processes such as wound healing, tissue remodeling and

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development of post-inflammatory fibrosis [11,12]. Eosinophils are weak antigen-presenting cells *in vitro*, suggesting that they may act as accessory cells in certain T cell-mediated immune reactions [13]. Furthermore, there is evidence that eosinophils slow the rate of progression of certain solid tumors through tumoricidal mechanisms [14]. Thus, at the moment the eosinophil is considered as a pluripotent cell with, depending on the circumstances, distinct roles including maintenance of normal tissue homeostasis, host defense, as well as propagation of tissue injury. It is worthwhile mentioning that, to date, the worldwide economic cost of one eosinophil-associated disease, bronchial asthma, is estimated to exceed the combined costs of the HIV/AIDS infection and tuberculosis [15].

2. Eosinophil Biology:

2.1 The normal mature eosinophil:

Eosinophils are nondividing, granule-containing cells [16]. They are end-stage cells derived from the bone marrow and mature under the influence of granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-3 (IL-3), and the terminal differentiation factor for eosinophils, IL-5 [17]. After a maturation time of 2-6 days, eosinophils efflux the bone marrow and circulate in the blood stream for 6-12 hours before migrating into the body tissues [18], where they survive for up to 2-3 weeks via autocrine production of GM-CSF [19]. It has been proposed that for every circulating eosinophil there are approximately 200 mature cells in the bone marrow and 500 in submucosal connective tissue throughout the body [18].

Eosinophils are approximately 8 μm in diameter with a bilobed nucleus, although three or more lobes are not uncommon. One of the most characteristic features of an eosinophil is its membrane-bound specific (crystalloid) granules of which there are about 200 per human eosinophil. The central core of these granules contains major basic protein (MBP) and is surrounded by a matrix composed of eosinophil cationic protein (ECP),

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eosinophil peroxidase (EPO) and eosinophil-derived neurotoxin (EDN or EPX) [20]. The granules are also the site of location of over 25 different cytokines and chemokines [16;21;22]. Like many other cell types, eosinophils contain lipid bodies which are the principal store of arachidonic acid and the enzymes cyclooxygenase and 5-lipoxygenase [16]. Eosinophil primary granules are a third type of intracellular organelle. They are recognized as distinct from specific granules by the absence of a core and are of variable size [23]. In normal peripheral blood eosinophils these granules are the exclusive storage site of Charcot-Leyden crystal protein (CLC protein), a lysophospholipase, [24]. Eosinophils, particularly those found in the peripheral tissues, also contain a number of small granules which stain intensely for acid phosphatase, aryl sulfatase and catalase [25,26]. Vesicotubular structures that are distributed throughout the cytoplasm contain cytochrome b_{558} [27]. This protein is a component of the superoxide (O_2^-) producing enzyme NADPH oxidase, and fuses with the cell membrane upon cell activation [28].

2.2 Eosinophil activation and mediator release:

Eosinophils from normal individuals circulate in a resting state, in which their effector functions and response to inflammatory mediators are blunted [29]. Once the eosinophil has migrated into inflamed tissue, it becomes activated. This results in release of stored proteins and *de novo* synthesis and secretion of mediators, allowing full expression of eosinophil effector functions [22]. The term "activation" has been used to describe this transition of an eosinophil from a resting to a primed and then secretory state [30,31].

Activated eosinophils form a spectrum ranging from primed cells with only subtle differences in effector function, as observed in eosinophils from the peripheral blood of subjects with allergic disease, to fully activated degranulating cells as found in biopsies of sites of florid eosinophilic inflammation such as asthmatic airways [32]. Morphological markers of activation include increased numbers and size of lipid bodies and increased numbers of primary granules, small granules, and vesicotubular structures [16]. Often, particularly in tissue eosinophils, there is a marked reduction in the number of specific

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granules and cells may appear necrotic (cytolytic) [33]. In studies conducted in airway eosinophils it has been shown that activated cells are characterized by increased O_2^- generation, enhanced adhesion to extracellular matrix (ECM) proteins, increased survival and rapid mobilization of intracellular Ca^{2+} stores [34].

It appears that eosinophils can release their granule contents through several mechanisms. The best characterized form of degranulation is exocytosis, also referred to as "*classical degranulation*" or granule extrusion. During this process, granules fuse with the plasma membrane and membrane-free specific granules are extruded [35]. This release mechanism occurs in human eosinophils *in vitro*. It has also been observed in human gut mucosa but not in airway tissue *in vivo* [36]. Alternatively, granules can fuse intracellularly into large degranulation chambers, which open to the outside of the cell through degranulation pores. This form of degranulation is called *compound exocytosis* [37], which is rare and appears to be associated with helminthic infection and cell activation by various drugs [38;39]. Another form of degranulation is a process known as *piecemeal degranulation (PMD)*. This term implies that granule protein-containing vesicles bud off from secondary granules resulting in their gradual emptying [40]. PMD of eosinophils commonly occurs *in vitro* and in diseased airway tissues *in vivo*. PMD has recently been quantified by determination of a degranulation index (PMDi) [41]. In humans, almost 70 % of eosinophils in inflamed airway tissue showed features of piecemeal degranulation compared to only 30 % necrosis (cytolysis) [41;42]. Moreover, it is important to keep in mind that quantitative ultrastructural measurements *in vitro* revealed a certain amount of PMD occurring during the purification procedure of eosinophils [43].

Once the eosinophils leave the circulation and migrate into the interstitial tissues or the lumina of the airways and the gut, many of these cells undergo *necrosis*. This mechanism of cytolysis is characterized by chromatolysis, centralization of granules and loss of plasma membrane integrity [16]. This process of granule release often occurs in human airway tissues *in vivo* [42]. The biology and pharmacology of induction and inhibition of eosinophil cytolysis remains to be explored. However, not all eosinophils found in the interstitium release their granule contents. Some of these cells retain their secretory mediators and simply undergo apoptosis. These cells are characterized by

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chromatin condensation, preservation of the plasma membrane, and non-dilated cell organelles [44]. Eosinophil apoptosis occurs both in airway lumina and *in vitro* [45]. Apoptotic eosinophils can commonly be detected in nasal discharge and sputum from asthmatics [46]. However, few investigators have yet succeeded in detecting apoptotic eosinophils in airway tissues *in vivo* [47], and there appears to be disagreement in the literature whether the data reported by Wolley and coworkers [47] actually constitute "compelling evidence" for the importance of apoptosis in airways *in vivo*. In summary, it may frequently be presumed that degranulation of eosinophils *in vitro* occurs via the classical degranulation mechanism. However, beyond a few *in vitro* studies describing either shape and membrane changes [48], or ultrastructural characteristics of exocytosis [35], little specific information about this mechanism is currently available. An interesting report suggests that eosinophil cytolysis is much more prominent *in vitro* than is the exocytosis mechanism [49]. If eosinophil exocytosis is a major mechanism in isolated eosinophils, this may be yet another example of differences between *in vitro* and *in vivo* models. Currently, a significant participation of classical eosinophil exocytosis remains to be demonstrated in diseased airway mucosae.

2.3 Eosinophil mediators:

Eosinophils have the capacity to synthesize and secrete a large number of potent inflammatory mediators. These include stored basic proteins in crystalloid granules, newly formed membrane-derived lipids, cytokines and chemokines, proteases, and products of oxidative metabolism [50]. Basic proteins include MBP, EPO, ECP and EDN [51]. These proteins are cytotoxic to mammalian cells as well as parasites, and their presence in the airway tissue may lead to bronchoconstriction and airway hyperresponsiveness [32]. Lipid mediators are oxidation products of arachidonic acid and include eicosanoids (leukotrienes, prostaglandins, thromboxans) and platelet activating factor (PAF) [52]. These mediators have a number of properties relevant to asthma such as

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smooth muscle contraction, mucus hypersecretion, and increased vascular permeability [53]. Eosinophils also synthesize and release up to 25 different cytokines, chemokines and growth factors including IL-1, -2, -3, -4, -5, -6, -8, 10, -12, -16, GM-CSF, tumor necrosis factor (TNF), RANTES and eotaxin [10;41;54;55]. IL-3, IL-5 and GM-CSF are particularly important in eosinophil maturation and differentiation [56], whereas IL-4 is crucial in IgE production in allergic individuals [57]. TNF is a potent proinflammatory mediator and its spectrum of activity ranges from cytotoxicity to eosinophil and other inflammatory cell activation, including mediator release and stimulation of oxidative metabolism [58]. RANTES, and to a lesser extent eotaxin, are chemokines produced by eosinophils and other cells, which lead to extravasation and accumulation of eosinophils at inflammatory sites [59]. Proteases produced by eosinophils include matrix metalloproteinases (MMPs: gelatinases, collagenases and stromelysins), histaminase, catalase, phospholipases and arylsulfatase [60]. MMPs are particularly important in extracellular matrix turnover and tissue remodeling and facilitate the transmigration of eosinophils through the vascular endothelial layer into the interstitial tissues [61]. However, the role of most other eosinophil proteases in health and disease remains uncertain.

Activated eosinophils can undergo a respiratory burst and generate considerable amounts of reactive oxygen metabolites, including O_2^- and H_2O_2 [62]. These highly reactive radicals are thought to be important in host defense mechanisms [63], cytotoxicity [64], bronchial hyperresponsiveness [65] and potentially as intracellular signaling molecules [66].

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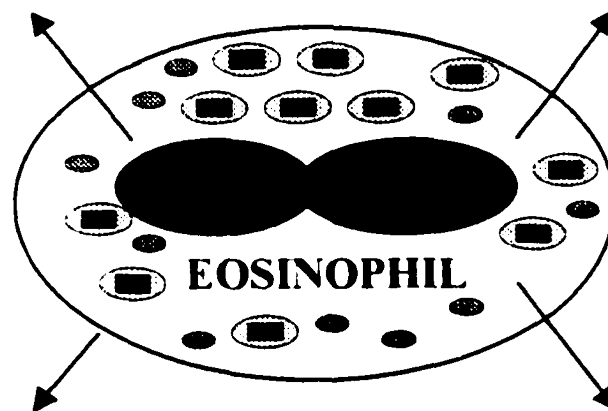
Fig.1.1

EOSINOPHIL MEDIATORS

Cationic Granule Proteins
Major Basic Protein
Eosinophil Cationic Protein
Eosinophil-derived Neurotoxin
Eosinophil Peroxidase

Cytokines, Chemokines and Growth factors, e.g.
IL-1α, IL-2, IL-3, IL-4,
IL-5, IL-6, IL-8, IL-9,
IL-10, IL-12, IL-16, TNF,
TGF-β, IFN-γ, GM-CSF,
RANTES, Eotaxin



Oxidative Metabolites
Reactive Oxygen Species
Nitric Oxide

***De novo* synthesized Lipid mediators**
LTC_4, PGE_2, PAF

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2.4 Mechanisms of mediator release:

The eosinophil is thought to be predominantly a secretory cell [16]. Although eosinophils are also capable of phagocytosis, this function appears secondary to that of mediator release. Of the many effects that can be elicited in eosinophils, perhaps the best understood are those processes controlling the regulated secretion of granule proteins, i.e. the phenomenon of exocytosis. Until relatively recently, the biochemical sequence of events that link activation of cell surface receptors to the expression of specific functional responses in eosinophils, also referred to as 'stimulus-secretion coupling", was a matter for conjecture. Secretion of granule proteins is thought to involve at least two steps: (i) mobilization and solubilization, which is followed by (ii) extracellular release [67].

In vitro, and possibly *in vivo*, eosinophil mediator release can be regulated selectively depending upon the nature of the stimulus. Substantive secretion of eosinophil granule proteins can be observed for example following interaction of the cell with large opsonized targets such as metazoan parasites or Sepharose beads [32]. In the case of parasite killing, cell adhesion to extracellular matrix proteins such as fibronectin, and immunoglobulin (Ig) binding to IgA- and IgG-receptors trigger degranulation and O_2^- production [68-70]. IgE-dependent stimulation was reported to induce the release of MBP and EPO but not ECP, while IgG-coated surfaces induce a selective release of ECP but not EPO [71]. Stimulation with soluble mediators such as GM-CSF and PAF results in degranulation in a CD11/CD18-dependent manner [72]. Furthermore, the interaction of agonists such as PAF and leukotriene B_4 (LTB_4) with their respective cell surface receptors has been shown to directly activate phospholipase C (PLC) [73]. PLC catalyzes the hydrolysis of phosphatidyl inositol-4,5-biphophspate (PtdIns-4,5-P_2) resulting in two second messenger molecules, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) [16]. Cytokines such as IL-3, IL-5 and GM-CSF are able to both prime eosinophils [74] and induce degranulation [43]. Signal transduction pathways involved in these responses include phosphorylation of Lyn and Jak2 tyrosine kinases, which bind to the common β-chain of the IL-5 receptor [75]. Activated Jak2 undergoes autophosphorylation and causes expression of nuclear transcription factors of the STAT family, especially STAT-1. IL-5 also stimulates the binding of GTP to p21 *ras*, which

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results in translocation of *raf-1* to the plasma membrane and its activation. *Raf-1* then phosphorylates MEK kinase, which subsequently activates MAP kinases[76]. Binding of TNF to one of the two TNF receptors (TNFR1 and TNFR2) described on eosinophils [77] causes activation of yet another pathway of intracellular signal transduction. The particularly complex mechanism of cellular activation employed by this cytokine acts via various intermediates including protein kinases, protein phosphatases, reactive oxygen intermediates, phospholipases, proteases, sphingomyelinases and transcription factors [78]. TNF receptor-associated factor-2 (TRAF2) mediates the signal from both TNF receptors to kinases of the MAP kinase family. The phosphorylation of these proteins activates IκB kinase and results in expression of the transcription factor NFκB [79]. On the other hand, phosphorylation of MAP kinases can activate Jun amino-terminal kinase (JNK) and p38 kinase cascades resulting in expression of the nuclear factor AP-1 [80].

Although different stimuli may activate different intracellular signaling mechanisms, almost all responses have one common step in their activation pathway, an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). A rise in $[Ca^{2+}]_i$ appears, therefore, to be prerequisite for exocytosis in secretory cells, but the source of Ca^{2+} and the mechanism by which it is mobilized, may vary depending upon the activation stimulus. Moreover, the mechanisms of Ca^{2+} mobilization may differ also from one cell type to another. For eosinophil exocytosis to occur, uptake of extracellular Ca^{2+} by the cell is believed to be supplemented by the intracellular release of Ca^{2+} induced by IP_3. It has been suggested that this pathway may require the activation of an as yet unidentified eosinophil Ca^{2+}-binding protein, together with a putative, membrane-associated, eosinophil protein (G_E) [81]. In guinea pig peritoneal eosinophils loaded with the fluorescent intracellular Ca^{2+} indicator fura-2/AM, the resting $[Ca^{2+}]_i$ is in the order of 120 nM and increases rapidly to about four fold values following the addition of a maximally effective concentration of PAF (1 μM) [82]. As in many other cells this effect of PAF is transient, peaking after 10-15 sec exposure, and returning to resting levels within 60 sec [82]. In addition to PAF, several other agonists increase $[Ca^{2+}]_i$ in eosinophils including LTB_4 and the complement fragment C5a, whereas the formylated bacterial tripeptide formyl-methionyl-leucyl-phenylalanine (fMLP) is only a very weak stimulant for Ca^{2+} mobilization, which is in contrast to its effect in neutrophils [83].

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Although intra- as well as extracellular Ca^{2+} stores are mobilized upon eosinophil activation, the exact contribution made by these two sources is in dispute. Kroegel et. al. [82] attributed the increase in $[Ca^{2+}]_i$ after activation with PAF predominantly to Ca^{2+} influx through, as yet poorly characterized, voltage-independent ion channels. In contrast, Minshall and coworkers [84] concluded from their studies in eosinophils that a considerable proportion of the Ca^{2+} mobilized by PAF originates intracellularly. Classically, intracellular Ca^{2+} can be released by IP_3 from the endoplasmic reticulum, or a specialized Ca^{2+} store, the so-called calciosome [85]. Other agonists including LTB_4, C5a and to a small extent fMLP, also promote the accumulation of IP_3 in eosinophils, whereas the phorbol ester PMA is inactive, indicating that protein kinase C (PKC) does not stimulate PLC [73].

Another requirement for eosinophil exocytosis appears to be the presence and availability of ATP. This is illustrated by the fact that in permeabilized cells ATP increases the affinities for both Ca^{2+} and GTPγS for their respective binding proteins [86]. ATP is also required to maintain the concentration of PtdIns-4,5-P_2 in cells and to provide, by nucleotide *trans*-phosphorylation, sufficient GTP for G protein activation. It is believed that ATP-driven phosphorylation reactions are essential for the early, but not later stages of the stimulus-secretion coupling mechanism. In contrast, GTP interactions with G_E are fundamental for the latter stages of regulated secretion of granule proteins in eosinophils [87].

3. Ion currents and cell activation:

3.1 Physiology of ion channels and electrical currents:

Ion channels are integral membrane proteins that provide low energy pathways for ions to cross the cell membrane. They allow ions to passively flow down their electrochemical gradients at rates exceeding 10^6 ions per sec. Ion channels can be characterized by their gating properties as well as their conductances, kinetics, ionic

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selectivity and pharmacology. Up to date, several types of ion channels have been described in inflammatory cells that open and close in response to specific chemical ligands (ligand-gated), voltage (voltage-gated), and other factors [88;89].

A net flow of charges caused by an electromotive force is called current (I) and is measured in Amperes (A). According to Benjamin Franklin, positive current flows in the direction of movement of positive charges. The size of a current is determined by two factors: the membrane potential (E_M) between the recording electrodes (measured in Volts; V), and the conductance (measured in Siemens; S). When 1 Volt is applied across a 1 Ohm resistor or 1 Siemens conductor, a current of 1 Ampere flows.

Much of what we know about ion channels was deducted from electrical measurements. Therefore, it is essential to remember certain rules of electricity before performing experiments. The most important one is *Ohm's law*, a relation between the parameters G, I and E_M [90]. G it is equal to I divided by E_M and its value is the inverse of the resistance (R) which is expressed in Ohms (Ω).

Ohm's law plays a central role in membrane biophysics because each ion channel is an

$$G = \frac{I}{E_M}[S] \qquad\qquad R = \frac{1}{G}[\Omega]$$

elementary conductor spanning the insulating lipid membrane.

Besides Ohm's law, another important electrical rule is described in the *Nernst equation* [91]. All biological systems are continuously moving towards a state of equilibrium, where the tendency for further changes vanishes. When applied to physiological solutions, the equilibrium potential (Eq) for a given ion is reached when the electrical force balances the diffusional, or chemical, force. Eq for each ion in solution is an important value in the description of the biological membrane potential of a cell. In an experimental setting, the applied voltage at which the concentration gradient is exactly opposed, is referred to as the "Nernst potential" (E_x). If we consider a mole of an arbitrary ion *x* with charge z_x, the Nernst equation is defined as follows:

$$E_x = \frac{RT}{z_x F} \ln \frac{[x]_o}{[x]_i}$$

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where E_x is the equilibrium or Nernst potential. R the gas constant, T the absolute temperature in degrees Kelvin. F the Faraday's constant, $[x]_o$ and $[x]_i$ the concentrations of the ion on the outside and inside of the cell membrane, respectively. This equation shows that there is an equivalency between chemical and electrical driving forces [92]. The Nernst equation allows the prediction of the membrane potential generated by one particular ion once its concentration gradient across the membrane is known. In physiological solutions, the equilibrium potentials for K^+ and Cl^- are negative, and for Na^+ and Ca^{2+} positive. In most biological systems, the equilibrium potential for K^+ sets the negative, and the one for Ca^{2+} the positive limit of the membrane potential. All cells have a negative resting membrane potential because at rest they have far more open K^+ and Cl^- channels than Na^+ or Ca^{2+} ones [88]. The Nernst equation is also useful to determine the selectivity of a channel. When a concentration gradient exists across a channel, current will flow until a sufficient voltage is applied to exactly oppose the current. If the required voltage is not the Nernst potential for any of the ions making up the gradient, it means that the channel is not perfectly selective for one ion but rather allows more than one type of ion to pass.

The application of Ohm's law and Nernst equation to a biological membrane reveals one of the central findings in cell electrophysiology: the electrical current of a particular ion equals zero at its equilibrium potential, and not at 0 mV. This finding is commonly graphically represented in form of a so-called current-voltage (I-V) relationship, where the current is depicted on the Y-axis and the voltage on the X-axis. An I-V curve contains information about the reversal potential of a certain current, which may help to identify the underlying ionic conductance, on ion channel parameters such as rectification, and on increases or decreases in current amplitude after activation or inhibition of a certain channel type, respectively.

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4. Electrophysiological measurements using the Patch clamp technique:

Currently, the patch clamp technique is a well-established method for measuring ion currents. This technique was originally developed by Neher and Sakmann in Goettingen with the intention of observing ion currents flowing through single ion channels in cell membranes [93]. Underlying all applications of patch clamping is the single principle that it provides an extremely sensitive means to measure ion currents flowing through cell membranes [94]. In fact, using this technique, ion currents of less than 1 pA (10^{-12} A) can be detected [95]. For their discovery and development of this technique and its application to a range of critical questions in biology, Neher and Sakmann were awarded the Nobel Prize in Physiology and Medicine in 1991 [96].

There are four basic patch clamp configurations [97]. Three of these are used to record single channel currents in membrane patches and the fourth is used to record currents through the whole-cell membrane:

- cell-attached patch
- inside-out patch
- outside-out patch
- whole cell recording

Before discussing these different configurations, one should be aware of certain universal electrical conventions used in patch clamp recordings. Firstly, the cell membrane potential is always expressed as the potential inside the cell with respect to a ground or zero level outside the cell. Secondly, by convention, positive ions flowing out of the pipette are measured as a positive current. Therefore, in the cell-attached configuration both Na^+ influx into the cell and Cl^- efflux results in a positive current. However, one has to be cautious when applying these rules to currents recorded in a patch clamp setting to not get confused with basic electrical conventions in biology, where a positive current is defined as a current of cations leaving a cell, e.g. a K^+ efflux or a Cl^- influx.

The formation of a high resistance seal between the patch pipette and the cell membrane is fundamental to all patch clamp recordings [98]. A problem in early patch clamp recordings were high noise levels. Neher and Sakmann found that the solution to

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the noise problem lied in the formation of a high-resistance seal between pipette and cell membrane. When precautions are taken to keep the pipette surface clean, tight seals with resistances of 10-100 GΩ can be obtained (so-called Gigaohm seal). To obtain such a high resistance seal, the pipette tip, usually 2-10 μm in diameter, is commonly fire-polished shortly before the start of the experiment. The Gigaohm seal is, therefore, highly effective in reducing the noise level of a recording and thus greatly increases the resolution of single channel currents [(97)].

The electrical resistance of the pipette is monitored by observing the size of a current pulse produced by a repeated test voltage pulse or "t-pulse". Most patch amplifiers have the facility to produce suitable pulses or they can be produced by a suitable pulse generator. Upon formation of a Gigaohm seal, the t-pulse disappears. A simplified schematic drawing of a patch clamp setup is shown in Fig. 2.1.

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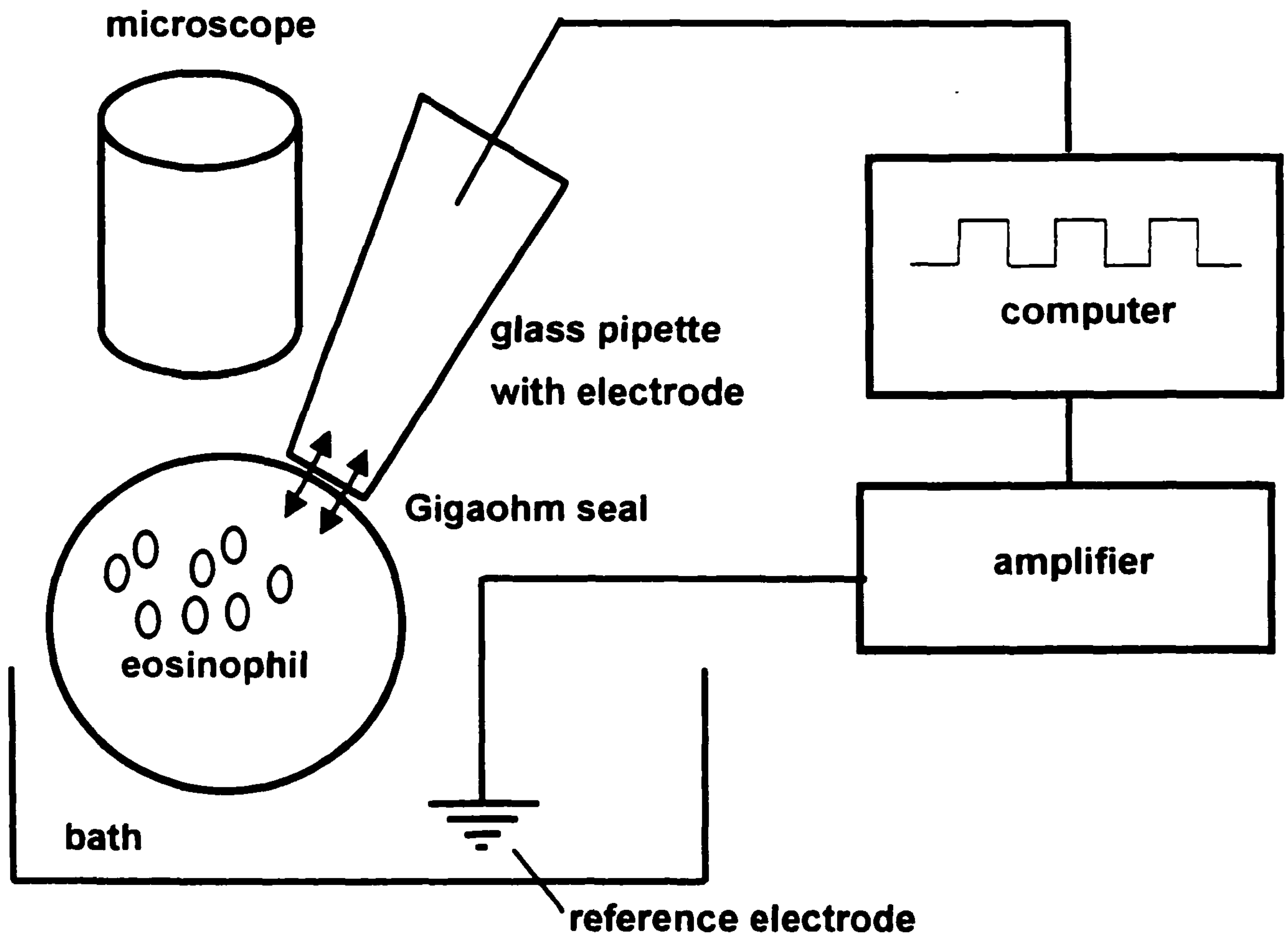
Fig.1.2

Patch clamp set-up



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The *cell-attached* patch is the simplest configuration and the one that probably causes least disturbance to the microenvironment of the ion channel [96]. In this configuration, single channels located directly under the pipette tip can be monitored. The membrane potential under the patch pipette equals the resting potential of the cell minus the potential applied to the pipette. This means that if the pipette potential is 0, the membrane potential of the patch equals the resting cell potential. When a negative current is observed, it can be interpreted as a cationic outward current, and a positive one as a cationic inward current. Since the integrity of the cell is maintained in this configuration, all biochemical mechanisms that may be linked to channel function, e.g. second messengers and protein kinases, are not disturbed. However, some inconveniences may be encountered using this configuration. The most important one is the inability to measure the real resting potential of the cell. Another problem is the inability to determine the driving force for a specific ion, which is defined as the membrane potential minus ionic equilibrium potential. This problem can be overcome by measuring the current at different applied membrane potentials and by blotting the current versus the voltage in an I-V relationship. Another strategy to overcome the unknown resting membrane potential is to simultaneously measure the membrane potential of the cell with an intracellular electrode. A third disadvantage is the impossibility to manipulate the pipette solution, unless a sophisticated perfusion system is used [99].

After obtaining a cell-attached configuration, there is a possibility to obtain a cell-free or excised patch. The easiest excised patch configuration to obtain is the *inside-out* patch. If the patch electrode is pulled away from the cell in the cell-attached configuration, the patch of membrane under the pipette tip is excised from the cell, but is still in place in the electrode and thus open to investigation. It is, therefore, possible to directly manipulate the internal membrane surface by modifying the composition of the bath solution, and also to control the potential across the membrane.

The second possibility after a successful cell-attached patch is to perforate the membrane under the patch to gain a direct communication between the pipette solution and the interior of the cell. In this *whole-cell* configuration, the patch electrode will record voltage or current changes from the whole cell membrane. An advantage of this

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technique is that it allows studying cells that are too small in size to be analyzed using intracellular microelectrodes. Since a direct communication is established between the pipette and the intracellular compartment, the cytoplasm is rapidly dialyzed by the pipette solution. This allows gaining control over the internal ion composition of the cell, second messengers and other soluble compounds [100]. This configuration has also been employed to measure exocytosis, since it allows continuous monitoring of the whole-cell membrane capacitance [94]. The whole-cell configuration can also be used to introduce enzymes such as the catalytic subunit of the cyclic AMP-dependent protein kinase, and other active protein agents such as GTP-binding proteins into mammalian cells. A drawback of this configuration is that during dialysis of the cytoplasm the intracellular material is extensively diluted. In addition, since the area of recordable membrane is quite large, the noise level is in often too high to discern single channel currents [98].

From the whole-cell configuration it is possible to achieve a new excised-patch. When the patch pipette is withdrawn very slowly, a small, circular membrane fraction is excised that spontaneously reseals on the pipette tip. This is referred to as *outside-out* configuration. The advantage of this configuration is that one is able to study single channel characteristics by manipulating the external surface of the membrane simply by adding pharmacological compounds to the bath solution.

A way to minimize the perturbation of the cytoplasm that occurs in the whole-cell and the outside-out configuration was devised by Horn and Marty [101]. In a so-called *perforated patch* clamp recording [102], the pipette tip is sealed to the cell membrane. The membrane under the patch is, however, not disrupted as in a whole-cell patch. Instead, a small amount of an ionophore such as gramicidin, nystatin or amphotericin B is added to the pipette solution to gain electrical access to the cell interior by forming small pores in the patch membrane [102;103]. These agents partition into lipids in the membrane and form tiny pores that allow exchange of monovalent cations and anions while excluding multivalent ions and nonelectrolytes the size of glucose or larger, including second messengers. Access resistance is an important parameter in the measurement of whole-cell recordings and is desired to be less than 20 MΩ. Therefore, many investigators choose amphotericin B over nystatin since amphotericin B channels have twice the single channel conductance of nystatin ones and thus produce lower access resistance [102].

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Patch clamp recordings can be performed in two different modes [88]:

- current clamp
- voltage clamp

In a *current clamp* experiment, one applies a known constant or time-varying current and measures the change in the membrane potential caused by the applied current. This type of current mimics the current produced by a synaptic input.

The *voltage clamp* technique was first invented by Marmot and Cole and further developed and exploited by Hodgkin and Huxley in 1952 [104;105]. In a voltage clamp experiment, one controls the membrane voltage and measures the transmembrane current required to maintain that voltage, even when membrane conductances are changing. In practical terms, an electronic amplifier compares the membrane potential with an applied potential, the "command potential". If a difference exists, the amplifier injects current into the cell proportional to that difference. The injected current brings the membrane potential as close as possible to the command potential. In this way, the membrane potential is controlled, or "clamped", to any desired value. There are certain advantages to perform such an experiment. Firstly, after each voltage step, the membrane potential is constant in time and voltage becomes an independent variable. Secondly, exept for the brief charging time, the currents flowing are proportional only to the membrane conductance. Limitations to the voltage clamp technique are the fact that the desired voltage cannot be established across the cell membrane instantaneously. When the membrane voltage is changed, the membrane capacitance is also changed, and this delays establishment of a constant potential. This might result in an initial short spike in a whole-cell recording, and is referred to as a capacitative current. Another limitation is that all parts of the membrane may not be held at the exact same potential. This arises from problems of spatial decrement of injected currents. Hence, the measured currents may be recorded from membrane areas that are not all at the same membrane potential.

Although the measurement of ion channel activity is one of the most common applications of the patch clamp technique, some investigators use this method to study the phenomenon of exocytosis. The underlying concept is the following: when a granule fuses with the plasma membrane during exocytosis, the area of the plasma membrane increases by the surface area of the granule in a single step. Such small changes can be

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resolved by membrane capacitance (C_M) measurements using the whole-cell configuration of the patch clamp technique [106]. When a secretory granule fuses with the plasma membrane, C_M increases by the capacitance of the granule membrane, leading to a capacitance step that reflects the size of the fusing granule. Therefore, this technique has been used in eosinophils to study mediator release. Generally, C_M is approximately 1 $\mu F/cm^2$ in all biological membranes. A resting eosinophil has a C_M of about 3 pF, corresponding to about 300 μm^2 of membrane area [48]. When eosinophils are stimulated with the membrane-permeable GTP analog GTPγS, a potent G protein-activating exocytotic stimulus, single granule fusion events were observed at low doses, whereas at high doses compound exocytosis appeared to prevail [48]. However, further studies will be necessary to determine whether different physiologic stimuli lead to different patterns of degranulation.

5. Ion channels in leukocytes:

Ion channels have been studied most extensively for over 50 years in the nervous system, where they function in information processing and signaling [107]. While ion channels in general, and voltage-gated channels in particular, were originally thought to be unique to neuronal cells, it is now evident that similar channels are present in most cell types. Their functions include establishing a resting membrane potential, shaping electrical signals, gating the flow of messenger Ca^{2+} ions, controlling cell shape and volume, and regulating the net flow of ions across cell membranes [108-110]. They also provide a crucial link between events occurring at the cell surface and a variety of cellular functions, including cell activation and mediator release [111;112]. It follows from studies in other cell types than neurons, that ion channels probably also play an important role in cellular physiology of leukocytes. Over the last decades, with the advent of the patch clamp technique developed by Sigworth and Neher [113], it has become feasible to record currents from small cells such as leukocytes, which had been difficult to study with intracellular microelectrode techniques. For this reason the area of leukocyte electrophysiology has progressed considerably in recent years. In addition, it became

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evident that leukocytes have extremely high input resistances in the order of 10^9 Ω, suggesting that their plasma membrane has a low permeability to ions [114]. Therefore, the opening of several channels, or even a single channel, on the surface of a leukocyte can produce a significant change in the membrane potential and conductance of the cell. The following pages will provide an overview of the most important classes of ion channels and summarize the literature on these channels in leukocytes.

5.1 Ca^{2+} channels:

By 1964, Hagiwara and his coworkers had undertaken extensive electrophysiological studies of Ca^{2+} action potentials and Ca^{2+} inward currents, initially in arthropod muscle [115]. Many features of Ca^{2+} channels were first revealed in this insightful comparative exploration. Using barnacle muscle, they learned that intracellular Ca^{2+} chelators favor excitability, that permeant divalent ions seem to compete for entry into the channel, and that divalent transition metal ions such as Ni^{2+}, Cd^{2+} and Co^{2+} block Ca^{2+} fluxes competitively. Nowadays, Ca^{2+} channels are recognized as both ubiquitous in nature and essential for various biological responses, ranging from muscle contraction to secretion. As a broad generalization, excitable cells translate their electrical potential into action via Ca^{2+} fluxes regulated by voltage-sensitive Ca^{2+} channels. Ca^{2+} ions are intracellular messenger molecules capable of activating a myriad of cellular functions. The biophysical properties of Ca^{2+} channels might have been determined by classical voltage clamp studies if the channels occurred in high density on a clampable membrane. However, these channels are rarely found in high density, and many of them occupy membranes that are difficult to clamp, such as dendrites, nerve terminals, and the complex infoldings of muscle cells [116-118]. Even when Ca^{2+} channels are on accessible surface membranes, their small currents are often masked by those of other channels, especially K^+ channels [119]. The ambiguity caused by these problems delayed the biophysical understanding of Ca^{2+} channels.

An increase in $[Ca^{2+}]_i$ can be the result of influx of extracellular Ca^{2+} or release of Ca^{2+} from intracellular stores. In excitable cells, voltage-gated Ca^{2+} channels (L-and N-

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type) regulate the entry of extracellular Ca^{2+} into the cytoplasm [120]. Other types of Ca^{2+} channels, the ryanodine and IP_3 receptor, regulate the release of Ca^{2+} from the endoplasmic reticulum, and are, to a much lesser extent, also found in the plasma membrane [121;122]. Interestingly, in nonexcitable cells, which often appear to lack voltage-gated Ca^{2+} channels, prolonged release from IP_3-sensitive stores can activate a poorly defined Ca^{2+} conductance at the plasma membrane [123]. In many cases, following cell activation, Ca^{2+} influx from the outside allows replenishing of intracellular Ca^{2+} stores after cell activation, and permits continuously stimulated cells to maintain elevated $[Ca^{2+}]_i$ levels even after depletion of their stores [124]. This Ca^{2+} inward current is referred to as Ca^{2+}-release-activated Ca^{2+} current (I_{CRAC}) and the responsible channel as store-operated Ca^{2+} channels (SOC) [88]. A typical manifestation of SOC occurs in the biphasic response to stimuli that generate IP_3 [125]. An initial transient rise in $[Ca^{2+}]_i$, which is thought to be the direct effect of such a stimulus, is commonly followed by a secondary peak in $[Ca^{2+}]_i$, which is believed to be the result of activation of SOC [124]. Finally, another Ca^{2+} permeable channel found in neurons, the NMDA receptor, is opened by the synaptic action of glutamate. Its main function is the voltage-dependent amplification of glutamatergic excitatory postsynaptic potentials (EPSPs) in the central nervous system [126].

Ca^{2+} fluxes play a vital role in leukocyte biology. It is well established that mitogens produce a rapid increase in $[Ca^{2+}]_i$, which is thought to constitute a mitogenic signal promoting T cell proliferation [127]. Stimulation of T cell activation by Ca^{2+} ionophores, e.g. A23187, the dependence of mitogen-induced proliferation on extracellular Ca^{2+}, and the demonstration of a mitogen-stimulated increase in cytosolic free Ca^{2+}, is consistent with this hypothesis [128]. Stimulation of T cells with another Ca^{2+} ionophore, ionomycin, was reported to stimulate IL-2 synthesis in T cells [129]. Proponents of this so-called "Ca^{2+} hypothesis" for T cell activation postulated the existence of Ca^{2+} channels to account for the mitogen-stimulated rise in cytosolic free Ca^{2+} in lymphocytes [130]. Although it was originally hypothesized that voltage-gated Ca^{2+} channels were responsible for the extracellular Ca^{2+}-dependent mitogen-induced rise in $[Ca^{2+}]_i$, it is now believed that they may not play a role in Ca^{2+} influx. This is because 1) they are absent in most T cells [114], 2) mitogen-induced Ca^{2+} increases are insensitive to

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blockers of voltage-gated Ca^{2+} channels [131], and 3) cell depolarization in a high K^+ medium, which is expected to increase Ca^{2+} influx through voltage-gated Ca^{2+} channels, has no effect on $[Ca^{2+}]_i$ [132]. This view is supported by two reports of voltage-insensitive Ca^{2+} channels in T lymphocytes [131;133], providing evidence for a non-voltage-gated Ca^{2+} transport process being responsible for the mitogen-induced Ca^{2+} influx. Other investigators suggested that T cell activation by mitogens resulted in an IP_3 increase that released Ca^{2+} from intracellular stores and also activated a transmembrane Ca^{2+} conductance in Jurkat T cells [134]. It should be noted, however, that for a long time non-voltage-gated Ca^{2+} channels have been described only in T cell lines. Therefore, until recently, the functional relevance of these channels in normal T cells was regarded as speculative. However, Densmore and coworkers described in 1996 a so-called voltage-operable Ca^{2+} current in human lymphocytes, where "voltage-operable" is defined as an intrinsic property of the channel protein rather than a requirement for of normal gating [135]. They found that inhibition of non-voltage-gated channels did not block either Ca^{2+} entry or Ca^{2+}-dependent lymphocyte proliferation, whereas blockade of this voltage-operable channel did. Therefore, the voltage-dependence of the rise in $[Ca^{2+}]_i$ upon T cell activation, remains controversial and needs to be further investigated.

T cell functions other than proliferation also appear to be regulated by Ca^{2+} fluxes. T cell-mediated cytotoxicity has been described to be Ca^{2+}-dependent [136]. Furthermore, a rise in $[Ca^{2+}]_i$ correlates with a shape change in cytotoxic T cells and a reorientation of cytoplasmic granules [137]. The Ca^{2+} influx that underlies antigen-mediated T cell activation has been solely assigned to activation of SOC [138]. Several other studies in T cells confirm the expression and physiological importance of SOC in lymphocyte activation [139;140]. In another mononuclear cell type, the monocyte, SOC has been shown to be differentially expressed during certain periods of cell growth and may regulate certain stages of cell maturation [141]. A role for another Ca^{2+} channel, the NMDA receptor, in the biphasic rise in $[Ca^{2+}]_i$ upon T cell activation was suggested by a study showing the expression of this channel type in natural killer (NK) cells, and its involvement in the recall of conditioned NK cell responses [142].

Similarly to lymphocytes, neutrophils exhibit a biphasic rise in $[Ca^{2+}]_i$ after cell activation [143]. This increase in $[Ca^{2+}]_i$ has been linked to differential secretion from the

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three granule populations of the neutrophil [144]. The early, transient rise in $[Ca^{2+}]_i$ was shown to be due to release of Ca^{2+} from intracellular stores, whereas the later in time and more sustained Ca^{2+} increase required extracellular Ca^{2+} and was attributed to a Ca^{2+} influx [145]. The existence of a Ca^{2+} influx pathway in neutrophils is suggested by the observations that chemoattractant-induced elevations in $[Ca^{2+}]_i$ are markedly decreased in the absence of extracellular Ca^{2+} [143], inorganic channel blockers such as Ni^{2+} and Cd^{2+} abolished the delayed phase of the chemoattractant–induced $[Ca^{2+}]_i$ elevation [145], and the readdition of Ca^{2+} after stimulation of neutrophils with an agonist in a Ca^{2+}-depleted medium led to an immediate $[Ca^{2+}]_i$ elevation [146]. In contrast to excitable cells, Ca^{2+} influx in neutrophils appears to be independent of plasma membrane depolarization and is, like in lymphocytes, insensitive to blockers of voltage-gated Ca^{2+} channels such as nifedipin [147]. Therefore, neutrophils are also thought to lack voltage-gated Ca^{2+} channels. Furthermore, chemoattractant-induced Ca^{2+} influx can be inhibited by pertussis toxin, suggesting that the mechanism of Ca^{2+} entry is coupled to the chemoattractant receptor via a G protein, either directly or via a second messenger [148]. Emptying of intracellular Ca^{2+} stores was reported to lead to extracellular Ca^{2+} influx and, via a hitherto unknown mechanism, to an increase in the plasma membrane permeability of neutrophils for Ca^{2+} [149]. Whether or not Ca^{2+} influx in neutrophils is, like in lymphocytes, caused by activation of SOC, remains to be shown. Evidence for the presence of such channels in neutrophils is suggested by a study showing that the second phase of an IL-8-induced biphasic increase in $[Ca^{2+}]_i$ was dependent on the presence of extracellular Ca^{2+} [150]. In spite of circumstantial evidence, the molecular confirmation of SOC in neutrophils remains a target for future experiments. In single channel patch clamp studies, Ca^{2+}-activated nonselective cation channels have been described [151], however, their physiological significance is not generally accepted [152]. Thus, the question whether Ca^{2+} enters the neutrophil through a Ca^{2+}-selective channel, a nonselective channel or some nonconductive transport pathway, remains open.

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5.2 K^+ channels:

Julius Bernstein first postulated in 1912 a selective K^+ permeability in excitable cell membranes and may be credited with opening the road to the discovery of K^+ channels [88]. In fact, in the following decades, K^+ channels of related structure have been found in prokaryotes and eukaryotes. Human K^+ channel genes can be classified into three groups based on the similarity in membrane topology [88]. The first group consists of channels characterized by 6 transmembrane domains (6TM), such as Ca^{2+}-activated (K_{Ca}) or voltage-gated (K_v) channels. The second group consists of channels with 4 transmembrane domains (4TM), such as two-pore-domain (K_{2P}) channels. The third group consists of channels with 2 transmembrane domains, such as ATP-dependent (K_{ATP}) channels or G-protein-coupled K^+ channels. The major role for K^+ channels consists in stabilizing the membrane potential. They draw the membrane potential closer to the equilibrium potential for K^+ ions. K^+ channels, therefore, set the resting membrane potential of a cell, keep fast action potential short by inducing repolarization, terminate periods of intense electrical activity, regulate the intervals during repetitive firing, and generally lower the effectiveness of excitatory inputs on a cell when they are open. On the other hand, closure of K^+ channels by second messenger systems is a mechanism to enhance excitability. In addition to these electrical roles, some K^+ channels have a transport role for salts and water across epithelial layers [153]. Probably all cells have K^+ channels, and the more channel types there are in one cell, the more difficult it is to distinguish their electrophysiological contributions. Employing the patch clamp technique, K^+ channels can be studied using pharmacological blockers and activators as well as by analysis of kinetic differences between the various families of K^+ channels. Single channel recordings are often useful because some K^+ channels have characteristic unitary current signatures and typical single channel conductances [154]. For many channels subtype-specific antibodies are commercially available nowadays and channels can be identified using molecular biological methodology. Although more than 80 mammalian genes for K^+ channels have been described, all K^+ channels share a common pore-lining P loop with a consensus amino acid sequence, also called the K^+ channel signature sequence [155].

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Voltage-gated K^+ channels (K_v) possess as their distinguishing feature an intrinsic voltage sensor able to detect the membrane potential [155]. These channels are composed of a type-specific α subunit that lines the pore, and up to three β subunits that associate with different α subunits [156].

Ca^{2+}-activated K^+ channels (K_{Ca}) are divided according to their conductances into channels of large (BK), intermediate (IK) and small conductance (SK). Whereas channels of large conductance are regulated by both voltage and Ca^{2+} and contribute to action potential repolarization, those of intermediate and small conductance are gated primarily by Ca^{2+} and are involved in generating the hyperpolarization after an action potential [157].

Inwardly-rectifying K^+ channels (Kir) exhibit strong interactions with the permeant ions and are well known for their ability to accommodate multiple ions in their pore [158]. The mechanism for inward rectification is accounted for primarily by a block of the channel pore by cytoplasmic cations [159]. Inwardly-rectifying K^+ channels are widely distributed in the mammalian brain and other tissues [160]. The different members of this family are regulated by a variety of intracellular signals such as hydrogen ions, redox reactions, protein kinases, and ATP hydrolysis [161].

ATP-sensitive K^+ channels (K_{ATP}) are composed of a pore-forming Kir6.x subunit and four β subunits, which form the sulfonylurea receptor (SUR) [162]. In pancreatic β cells, these channels regulate insulin secretion [163], whereas in the heart they mediate cardioprotection during ischemia and cardiac arrhythmia [164].

In human proliferating lymphocytes, only one type of voltage-gated K^+ channels, namely n type (K_n) or $K_v1.3$, has been described. In contrast, three voltage-dependent K^+ conductances have been delineated in murine T cells [114]. The K_n channel is one of the best-characterized ionic conductances in leukocytes. Whole-cell patch clamp experiments revealed a threshold of activation in the range of −50 to −60 mV, and its conductance is fully activated above 0 mV. These currents exhibit the typical sigmoidal activation kinetic of voltage-activated K^+ channels [89]. Furthermore, K_n channels are inactivated by increases in $[Ca^{2+}]_i$ [165], and by a decrease in temperature [166] and intracellular pH [167]. Both, K^+ channels blockers such as quinidine, TEA and 4-AP, and Ca^{2+} channel blockers such as diltiazem, verapamil and nifedipin, are described to block the K_n channel [89]. The total number of voltage-gated K^+ channels in human lymphocytes is upregulated by

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approximately 70% following mitogen stimulation [168]. An increased number of K^+ channels can also be induced following stimulation of lymphocytes with IL-2, and is reversible upon removal of this stimulus. This accompanies the return of the cells to their resting state [169]. T cell stimuli such as concavalin A and phorbol esters change the voltage dependence of K^+ channels so that they open more readily upon depolarization [170]. Killing of allogeneic target cells by cytotoxic T cells is also inhibited by K^+ channel blockers [171]. Furthermore, most but not all protein synthesis, including IL-2 production, is inhibited by the broad spectrum K^+ channel blockers TEA and 4-aminopyridine (4-AP) [168].

The presence of K^+ channels in neutrophils has been suggested by analysis of their membrane potential. The resting membrane potential of neutrophils was estimated to be around -60 mV, with more positive values as the extracellular K^+ concentration is increased. This suggests that neutrophils possess K^+ channels that maintain the negative membrane potential of the cell [172]. Similarly to lymphocytes, K_v channels have been described in unstimulated neutrophils, with a threshold of activation of –60 mV. These currents showed inward rectification and were blocked by Ba^{2+} [148]. Evidence for the presence of K_{Ca} channels in neutrophils is suggested by studies showing that ionomycin induced an increase in K^+ outward current [173]. In addition, exposure of HL-60 promyelocytic cell line to ionomycin produced membrane hyperpolarization, and using ion substitutions and ion channel blockers, it has been shown that this hyperpolarization was mediated by K_{Ca} channels [174]. In conclusion, it appears that K_v channels are important for the maintenance of the resting membrane potential of leukocytes, whereas K_{Ca} channels regulate the repolarization after cellular activation [148].

5.3 Cl^- channels:

Cl^- is by far the most abundant physiological anion and plays an important role in cellular homeostasis. In most cells it is distributed close to its equilibrium. The equilibrium potential for Cl^-, E_{Cl}, is usually near the resting membrane potential [88]. Therefore, similar to K^+ channels, Cl^- channels are thought to oppose normal excitability.

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to stabilize the membrane potential and, in part, help repolarize an activated cell. In addition, Cl^- anions play a role in intracellular pH regulation, cell volume regulation and as a driving force for secretion of fluid from glands and epithelia [175-177]. The first Cl^- channels were discovered as so-called "background channels" in vertebrate twitch muscle by Boyle and Conway in 1941 [178]. This model is still the best-studied example of an excitable cell with a high resting Cl^- permeability. The voltage- and time-dependence of this Cl^- conductance was described as mild and slow [179]. Therefore, in many patch clamp recordings of fast channels, Cl^- currents are often masked by leak currents. However, distinct Cl^- channels can be identified using pharmacological blockers and anion substitution.

Four well-established classes of Cl^- channels are now recognized: the cAMP-activated cystic fibrosis transmembrane conductance regulator (CFTR) channel, the voltage-gated Chloride Channel (ClC) family, ligand-gated Cl^- channels (γ-aminobutyric acid (GABA) and glycine receptors) and Ca^{2+}-activated Cl^- channels (CaCCs) [180;181]. Genetic defects leading to inherited disease are known for the first three of these classes [182]. A combination of mutagenesis and biophysical analysis has been used to correlate their structure with function.

The CFTR Cl^- channel belongs to the class of ATP-binding cassette transport ATPases and is one of the best-studied Cl^- channels. It is composed of 12 transmembrane helices in two groups of six, two cytoplasmic nucleotide binding domains (NBDs), and a cytoplasmic region, the regulatory or R domain, containing numerous consensus sequences for phosphorylation [183]. Currently, it is well established that CFTR is a cAMP–dependent channel and is acutely regulated at its R domain by protein kinases, particularly PKA, and by phosphatases [184;185]. Phosphorylation by cAMP-dependent protein kinase activates the channel. Binding and hydrolysis of ATP at the NBDs regulates channel opening and closing, whereas the R domain plays an important role in coordinating the open and closed states of the channel [186]. Channel gating kinetics are characteristically slow with long closed periods and little voltage-dependence [187]. In addition to transport of Cl^- ions, CFTR may also be a regulator of other ion channels. It has been shown that patients with cystic fibrosis show an increased Na^+ reabsorption across lung epithelia, suggesting that CFTR may regulate the epithelial Na^+ channel

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(ENaC). Indeed, coexpression of CFTR with ENaC demonstrated that it inhibits this Na^+ channel in a cAMP-dependent manner [188]. Other data suggest that CFTR may regulate the activity of the renal K^+ channel ROMK2 [189].

By expression cloning in *Xenopus* oocytes, Jentsch and coworkers discovered the first member of the voltage-gated ClC family, ClC-0 [190]. Subsequent homology-based cloning revealed that ClC-0 is a member of a large gene family with nine mammalian genes recognized [191]. In mammals, the different genes are expressed in different cell types. Some isoforms are expressed predominantly in one cell type (e.g. ClC-1 in skeletal muscle) or in one organ (e.g. ClC-Ka and ClC-Kb in kidney), whereas others appear to be more widely distributed (e.g. ClC-2, -6, -7) [192]. The transmembrane topology of ClC channels is still largely conjectural, since most studies were performed using hydropathy analysis. This analysis, when applied to ClC-0, led to the prediction of 12-13 transmembrane spans [190]. ClC channels probably function as dimers, and single channel recordings of ClC-0 suggest a peculiar double-barreled structure of this channel [193]. Furthermore, studies of glycosylation revealed that ClC-0, -1, -2 and –K are glycosylated [194]. Electrophysiological characteristics of ClC channels are a high selectivity for Cl^- anions and a voltage-dependent block by I^-. [195] They display a rather small single channel conductance, which is largest for ClC-0 with about 9 pS [196]. The physiological functions are clearest for ClC-1 (control of muscle excitability) and ClC-2 (cell volume control), but are still obscure for newer family members which often cannot yet be reliably expressed as Cl^- channels [197].

The third group of Cl^- channels, ligand-gated Cl^- channels, include the γ-aminobutyric acid (GABA) and the glycine receptor. These are postsynaptic Cl^- channels that are predominantly expressed in neuronal tissue [198]. Opening of these channels is mostly inhibitory, since the resulting stabilization of the voltage counteracts depolarization caused by excitatory neurotransmitters. GABA and glycine receptors are oligomeres composed of several identical subunits and are believed to function as pentameric complexes. The single subunits have four membrane-spanning domains and the second transmembrane domain M2 forms the pore [182]. Both GABA- and glycine-gated anion channels have multiple conductance levels in the 10-90 pS range [199]. GABA receptors are divided into $GABA_A$, $GABA_B$ and $GABA_C$ subtypes. Only $GABA_A$ and

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$GABA_C$ receptors are believed to represent Cl^- channels. $GABA_A$ receptors are selectively blocked by the alkaloid bicuculline and are modulated by benzodiazepines, steroids and barbiturates. In contrast, $GABA_C$ receptors are not blocked by these drugs, but are sensitive to (1,2,5,6-tetrahydrpyridin-4-yl)methylphosphinic acid (TPMPA) [200]. $GABA_B$ receptors activate the second messenger systems PLC and AC, and activate K^+ and Ca^{2+} channels via G-coupled proteins. These receptors produce slow, prolonged inhibitory signals and function to modulate the release of neurotransmitters [201].

Ca^{2+}-activated Cl^- channels (CaCCs) were first described by Ran and coworkers in 1992 [202]. These channels have a single channel conductance of about 25 pS and show an ion selectivity of $I^- > Cl^-$, which is the opposite of CFTR and ClC-2 [203]. CaCCs seem to be primarily regulated via Ca^{2+}-dependent protein kinases and appear insensitive to regulation by PKA. They are activated and phosphorylated by calmodulin-dependent kinase II (CaMK II) and by PKC. They are composed of four transmembrane domains with several consensus sites for glycosylation. This group attracted particular attention when experiments in cystic fibrosis knock out mice showed that CaCCs were capable of upregulating Cl^- secretion in the epithelium [204]. Although the CaCCs conductance pathway is conserved in humans, for unknown reasons these channels are not able to effectively substitute for CFTR.

In T and B lymphocytes, single channel currents of a large-conductance and voltage-dependent Cl^- channel have been described [171;205]. The single channel conductance of this channel was about 365 pS with several subconductance states. The channel opened and closed in a time-dependent manner, was temperature-sensitive and blocked by Zn^{2+} [206]. In addition, in human T cells a Cl^- channel of small conductance has been described (Cl_s). The currents were too small to resolve at single channel level, but noise analysis resulted in an estimate of 2.6 pS for their single channel conductance. Hydrolysis of ATP was required for activation and it was hypothesized that Cl_s channels participate in lymphocyte volume regulation [89].

In neutrophils, Cl^- efflux is an early event occurring after exposure of these cells to agonists such as TNF and GM-CSF [207]. A possible relationship between Cl^- efflux, adherence and respiratory burst was first suggested by kinetic studies, showing that TNF-induced Cl^- efflux preceded both the adhesive and the metabolic response, and was then

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confirmed by inhibition of all three responses with Cl^- channels inhibitors such as ethacrynic acid [208]. Other investigators described both voltage-independent Ca^{2+}-activated [173] as well as voltage-dependent Cl^- currents in human neutrophils [209]. These channels are believed to participate in neutrophil activation, volume regulation, secretion, cytotoxicity and proliferation. The induction of voltage-dependent Cl^- currents in neutrophils by phorbol esters was associated with activation of PKC. Other Cl^- currents in neutrophils were found to be activated in hypotonic solution [210]. These outwardly-rectifying Cl^- currents displayed no apparent voltage- or time-dependence and appeared to be induced by membrane stretch. The single channel conductance of these channels was 1.5 pS, which led to the term "mini-Cl^- channels". Currents with very similar characteristics were also described in the neutrophil HL-60 cell line [211]. Biochemical and ultrastructural studies performed by Korchak and coworkers in 1982 showed that the Cl^- channel blockers 4-acetamido-4-isothiocyano-2,2-disulfonic acid (SITS) and 4,4-diisothiocyano-2,2-disulfonic acid (DIDS) specifically inhibited fusion of lysosomes with the plasma membrane of neutrophils. However, ion substitution studies indicated that influx of Cl^- was apparently not critical for these events which appeared to occur independently of the extracellular Cl^- concentration [212], suggesting that these drugs may act on a target different than Cl^- channels. Interestingly, in contrast to neutrophil degranulation, O_2^- production was not inhibited by these Cl^- channel blockers [213].

5.4 H^+ channels:

Proton (H^+) channels were first discovered by Thomas and Meech in snail neurons during studies of acid regulation [214]. Neurons were voltage clamped while acid loads were injected and the intracellular pH was measured. The H^+ channels carried outward fluxes of H^+ when the membrane was positive to the equilibrium potential of H^+, and helped to remove excess acid from the cytoplasm. H^+ channels activate with depolarization like a voltage-gated, slow delayed rectifier, but in a physiological pH range their unitary conductance is often below the resolution of direct measurement.

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An essential physiological function of voltage-gated H^+ channels is to aid the production of O_2^- in phagocytes such as neutrophils, macrophages and eosinophils via a reaction called the respiratory burst [215]. This reaction is elicited by the NADPH oxidase, includes the transfer of electrons from the inside to the outside of the cell, and the generation of H^+ ions in close proximity to the cytosolic face of the plasma membrane. In order to avoid excessive acidification of the cytosol, H^+ ions need to be extruded. H^+ transport through H^+ selective channels has been proposed to achieve this goal [216]. However, this suggestion was based on indirect studies using fluorescent dyes, and no actual current measurements were performed. In the following years, H^+ currents were recorded from human neutrophils and HL-60 cells using the whole-cell patch clamp technique. This method allowed the detection of voltage-activated currents that were carried by H^+ ions, since they occurred in the absence of permeant ions other than H^+ and followed the electrical gradient of H^+ [217]. The H^+ currents were activated by depolarizing voltages, were gated by the intracellular and extracellular pH and were remarkably similar to the currents described Thomas and Meech in snail neurons [214]. In the search for the identity of this H^+ conductance, it was proposed that an NADPH subunit, gp91 *phox*, may function as a voltage-dependent H^+ conductance [218]. The authors of this study described the observed H^+ currents as time-dependent and Zn^{2+}-sensitive but insensitive to the extracellular Cl^- concentration. Evidence for gp91 *phox* being an integral part of the H^+ conductance is further provided by studies showing that in neutrophils from patients suffering from chronic granulomatous disease (CGD), which lack this subunit, no H^+ currents could be induced [219,220]. In contrast, the absence of another NADPH oxidase subunit in neutrophils from CGD patients, cytochrome *b*, had no effect on induction or amplitude of the H^+ current [221].

Several agents known to activate leukocytes such as PMA, TNF and fMLP have been shown to activate the NADPH oxidase and associated H^+ currents. In neutrophils, the addition of PMA caused a negative shift in the H^+ channel I-V relationship, faster activation during depolarization, slower inactivation during repolarization and larger maximum current amplitude [222]. Simultaneously, an inward current that directly reflected electron transport by the NADPH oxidase was also activated by PMA. The identity of this electron current was confirmed by its sensitivity to diphenylene iodonium

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(DPI), an inhibitor of the NADPH oxidase. Similarly to PMA, neutrophil stimulation with TNF or fMLP also evoked similar voltage-dependent H^+ currents [223].

6. Ion channels in eosinophils:

Compared with other leukocytes, very little is known about expression and function of ion channels in eosinophils. Although it seems reasonable to assume that eosinophils express Ca^{2+} channels, the evidence for their existence is, to date, mostly circumstantial. An increase in the $[Ca^{2+}]_i$ concentration is commonly accepted as a prerequisite for eosinophil activation and mediator release [112]. Nevertheless, at present, no reports have convincingly shown the expression of voltage-gated Ca^{2+} channels in eosinophils. Evidence for the presence of these channels is suggested by a study showing that stimulation of eosinophils with antigen caused an increase in O_2^- and LTC_4 release, which were inhibited by the voltage-gated Ca^{2+} channel blocker verapamil [224]. Furthermore, using monoclonal antibodies, IP_3 receptors were found in eosinophils [225]. Upon stimulation with chemoattractants, eosinophils show a rapid increase in $[Ca^{2+}]_i$ that is insensitive to removal of extracellular Ca^{2+}. Blocking of IP_3 receptors prevented the rise in $[Ca^{2+}]_i$ suggesting that it is produced by discharge from internal stores [226]. In contrast, in another study, the rise in $[Ca^{2+}]_i$ induced by PAF was shown to be dependent on the extracellular Ca^{2+} concentration. The authors conclude that Ca^{2+} entry via receptor-operated Ca^{2+} channels may be involved in PAF-induced eosinophil degranulation [82]. Finally, it has been shown that eosinophil O_2^- production upon stimulation with PAF and fMLP required intra- as well as extracellular Ca^{2+}, since removal or chelation of either one resulted in decreased O_2^- release. However, the authors of this study failed to identify the transport mechanism underlying the increase in $[Ca^{2+}]_i$ [227]. At present, there is no information available on the expression of SOC in eosinophils, and therefore, the involvement of I_{CRAC} currents in Ca^{2+}-induced responses in these cells, remains a matter of speculation.

Most studies of ion channels in eosinophils were performed on K^+ channels. A role for K^+ channels in eosinophil biology was first suggested by reports showing that K^+

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channel blockers such as quinidine, sparteine and 4-AP inhibited cell shrinkage during apoptosis. This effect was attributed to inhibition of K^+ efflux from the cell [228]. At the same time, other investigators showed that quinidine inhibited O_2^- production in patients with hypereosinophilic syndrome, and proposed a role for K_{Ca} channels in this process [229]. Further evidence for these channels being present in eosinophils was provided by studies indicating that the eosinophil-specific chemokine RANTES activated K^+ channels in a similar manner as did the Ca^{2+} ionophore A23187 or an increase in $[Ca^{2+}]_i$. Similar results were obtained using PAF [230]. A few years later, Saito and coworkers found a correlation between activation of K_{Ca} channels and eosinophil mediator release. The K^+ channel blocker quinidine inhibited both the release of MBP and K_{Ca} channels in human PAF-stimulated eosinophils [231]. A role for K^+ channels in eosinophil survival and O_2^- production was suggested by studies showing that sulfonylureas, inhibitors of K_{ATP} channels, induced eosinophil cell death and inhibited O_2^- generation. The authors of this study also showed mRNA expression of a K_{ATP} channel subunit, the sulfonylurea receptor, in human eosinophils [232]. In addition, Tare and coworkers described a whole-cell inward current in human eosinophils, which was activated upon membrane hyperpolarization and blocked by Cs^+ and Ba^{2+}. This current was attributed to the presence of inward rectifier K^+ channels. The authors exclude a role of these channels in O_2^- production but suggest an involvement in setting the membrane potential of eosinophils close to the equilibrium potential for K^+. Molecular biological studies identified this channel as Kir2.1 [233].

Very little information is available on Cl^- channels in eosinophils. Nedocromil sodium and sodium cromoglycate, two anti-asthma drugs and blockers of Cl^- and Ca^{2+} channels [234], were found to inhibit the PAF-induced increase in eosinophil cytotoxicity against parasites [235]. In allergic disease, the mode of action of these drugs is thought to be downregulation of eosinophil and neutrophil [236], but not lymphocyte [237], activation, while stabilization of mast cell membranes appears negligible. This effect may be mediated via inhibition of Cl^- channels [238;239]. Another study showed that two Cl^- blockers, DIDS, a stilbene disulfonate, and NPPB, a derivative of diphenylamine-2-carboxylic acid, inhibited LTB_4-induced O_2^- production. However, removal of extracellular Cl^- had no effect on the respiratory burst. The authors concluded that DIDS

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and NBBP were not inhibiting the LTB_4-induced response by blocking Cl^- influx. Nevertheless, when eosinophil O_2^- production was induced with opsonized zymosan, the presence of extracellular Cl^- was found to be an absolute requirement [(240)].

Similarly to neutrophils, eosinophil H^+ channels have been involved in activation of NADPH oxidase and respiratory burst. The first H^+ currents in eosinophils were described as both voltage- and pH-dependent. The currents were activated by depolarizing voltages, low pH and increased $[Ca^{2+}]_i$, and were blocked by Zn^{2+} and Ni^{2+}. The density of the H^+ conductance in human eosinophils is with 1.5 ± 0.1 nS/pF among the highest that has been reported [(241;242)]. Banfi and coworker were able to differentiate two different H^+ conductances in human eosinophils. The previously described "classical" H^+ current was present in CGD eosinophils, whereas a "novel" H^+ conductance was absent in cells from patients deficient in gp91 *phox* and p47 *phox*, two subunits of the NADPH oxidase. Unique properties of this new channel when compared with the "classical" channel were its low threshold of voltage activation allowing H^+ influx and intracellular acidification, its faster activation and slower deactivation, its 20-fold higher sensitivity to Zn^{2+} and the fact that it was only observed upon NADPH oxidase activation [(243)]. These properties led the authors to conclude that the physiological function of this novel H^+ conductance may not be restricted to H^+ extrusion and repolarization, but could include depolarization, pH-dependent signal termination, and determination of the phagosomal pH set point. However, a recent study by De Coursey and coworkers argued against the presence of two different H^+ conductances in eosinophils on the basis that in their hands unstimulated and stimulated eosinophils displayed similar sensitivity to Zn^{2+}. These authors propose a single type of H^+ channel with properties that can be modulated by stimuli such as PMA [(244)].

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OBJECTIVES AND HYPOTHESES

This thesis dealt with two aspects of the role of eosinophils in inflammatory responses associated with allergic inflammation. First, the recruitment of eosinophils to peripheral tissues and the mechanisms that need to be in place to allow eosinophil migration to the sites of inflammation. Matrix metalloproteinases (MMPs) are crucial enzymes in eosinophil extravasation and transmigration into the airways [(245)]. In particular, the gelatinase MMP-9 is capable of degrading collagen type IV, a major constituent of basement membranes [(246)]. Activation of this enzyme is, therefore, thought to promote eosinophil recruitment into the airway mucosa and to facilitate their transmigration into the airway lumen. In addition to their contribution to acute inflammatory processes, MMPs may participate also in more chronic inflammatory events by regulating tissue remodeling [(247)]. The balance between MMP-dependent extracellular matrix degradation and tissue inhibitors of matrix metalloproteinases (TIMP)-dependent fibrosis appears crucial in the final outcome of airway inflammation.

The second aspect of this thesis focused on the fate of eosinophils once they infiltrated the inflammatory environment of the airways. When expose to such a milieu, eosinophils are activated and upregulate their mediator release. In this part of the study I measured eosinophil activation as an increase in O_2^- production and investigated its regulation by ion channels. This section of the thesis also addressed the regulation of eosinophil ion channels by reactive oxygen metabolites, in particular nitric oxide (NO), which is present at elevated concentrations in the exhaled air of asthmatics [(248)]. In summary, this study tried to characterize (i) mechanisms employed by eosinophils to infiltrate the airway mucosa and (ii) the regulation of eosinophil activation as measured by O_2^- production and whole-cell current activation.

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Therefore, the objectives of this study were

1) to investigate the mechanisms of eosinophil mediator release, particularly MMP-9 and O_2^-
2) to identify and characterize membrane ion channels and other transport proteins involved in human eosinophil activation
3) to determine the effect of reactive oxygen species on human eosinophil ion channel function

We hypothesized that

1) cytokines present in an inflammatory environment regulate MMP synthesis and release from human eosinophils
2) Ca^{2+}, K^+, Cl^- and H^+ channels as well as other membrane transport proteins (e.g. Cl^-/HCO_3^- exchanger) play a role in human eosinophil activation and mediator release
3) increased amounts of reactive oxygen metabolites, as found at inflammatory sites, alter eosinophil ion channel function

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LEGENDS TO FIGURES

Fig.1.1

Simplified schematic illustration of a selection of eosinophil-derived mediators. Eosinophils synthesize, store and release cationic granule proteins, oxidative metabolites, as well as numerous cytokines chemokines and growth factors. Lipid mediators are *de novo* synthesized in eosinophils and may be derived from membrane phospholipids or intracellular lipid bodies.

Fig.1.2

Simplified schematic drawing of a patch clamp set up in the whole-cell configuration. Computer-generated signals are transmitted to the cell under investigation via the pipette electrode. Currents flowing through the cell membrane are recorded with reference to the bath electrode, transmitted to an amplifier, and can then be analyzed using appropriate computer software.

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CHAPTER 2

Human eosinophils release matrix metalloproteinase MMP-9 upon stimulation with tumor necrosis factor-α.

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INTRODUCTION:

A precise role for the eosinophil in inflammatory diseases has remained elusive [1]. Eosinophils are thought to exert a beneficial function in protecting the host from parasitic infection [2], while at the same time they are considered to play a detrimental role in a variety of acute and chronic airway disorders. In bronchial asthma, strong evidence has been provided to indicate that eosinophils may be responsible for damage to the bronchial epithelium by releasing cytotoxic granule proteins [3] and other proinflammatory mediators [1,4]. The number of eosinophils is also increased in both peripheral blood and airways of atopic and non-atopic asthmatics [5]. Furthermore, the concentration of eosinophil products has been shown to correlate with disease severity [6]. Nevertheless, evidence for an effector function of the eosinophil remains circumstantial. This fact is complicated by the presence of a mixed inflammatory reaction involving neutrophils [7], macrophages [8], lymphocytes [9], mast cells [10], and eosinophils [11].

At the histopathological level, one characteristic of asthmatic tissue is sub-basal membrane thickening and hypertrophy as well as hyperplasia of smooth muscle cells and subepithelial glands [12]. These features are thought to correlate directly with the activity of matrix metalloproteinases (MMP) in the airway interstitial tissue, including MMP-9 [13]. Abundant expression of MMP-9 mRNA has been detected in airway biopsies from asthmatic subjects. Interestingly the vast majority of cells expressing MMP-9 mRNA were eosinophils [14]. Asthmatic tissue associated with the expression of MMP-9 was characterized by remarkable inflammation, including mucosal edema, cellular infiltration, and expression of adhesion molecules [12]. These findings suggest the overexpression of MMP-9 by eosinophils in bronchial tissue of asthmatic individuals, and the participation of MMP-9 in the maintenance of airway inflammation in asthma.

Among the cytokines involved in MMP regulation [13], there is evidence to suggest that TNF-α may be crucial, particularly with MMP-9. In fact, TNF-α has been demonstrated to markedly upregulate MMP-9 production in human monocytes [8]. These data have been confirmed in endothelial cells [15], fibroblasts [16] and leukemia cells [17]. TNF-α effects in human inflammatory cells are regulated via specific receptors, TNF-αRI or TNF-αRII. Eosinophils express at least one of the two receptors [18].

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It is well known that not only cytokines such as TNF-α regulate MMP-9 activity, but also the physiological tissue inhibitors of matrix metalloproteinases, TIMP-1 and TIMP-2 [19]. TIMP-1 seems to be closely associated with MMP-9 activity [13] although this has not yet been confirmed in eosinophils. Interestingly, in human monocytes TIMP-1, but not TIMP-2 expression was shown to be induced by TNF-α [8]. Although the stimulatory effect of TNF-α on MMP-9 appears to be consistent, its effect on TIMPs needs further investigation.

In this study we hypothesized that in eosinophils 1) TNF-α is a major activator of MMP-9 activity, and 2) TNF-α influences the physiological equilibrium between MMP-9 and TIMPs. In order to understand the equilibrium between MMP-9 and TIMPs upon stimulation with TNF-α we examined the expression of MMP-9 and TIMPs and the amounts of MMP-9 and TIMP proteins produced by peripheral blood eosinophils. Our data suggest that TNF-α markedly increases MMP-9 release from human eosinophils. Furthermore, the production of inhibitors of MMPs appears inconsistent among different atopic subjects. These results have important implications on the potential role of the eosinophil in airway remodeling in asthma.

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MATERIALS AND METHODS:

A) Isolation of peripheral blood eosinophils:

Peripheral blood eosinophils were isolated from atopic asthmatic volunteers who had given their informed consent, as follows [20;21]. Heparinised blood (100 mL) was mixed with 20 mL Dextran (6 %) (Fluka, Buchs, Switzerland) in RPMI-1640 (Bio Whittaker, Maryland, USA). Erythrocytes were then sedimented for 30 min at room temperature. The plasma was layered on a Ficoll (Pharmacia, Quebec, Canada) cushion (15 mL) and centrifuged for 25 min at 1000 g at room temperature. Contaminating erythrocytes in the resultant pellet were lysed in 2 mL sterile water for 5 sec and the cell suspension was then washed in RPMI-1640. The pellet (containing approximately 5×10^7 cells) was resuspended in 600 µL RPMI-1640 containing 2 % FCS/0.5 mM EDTA (Sigma, St. Louis, USA) and incubated for 45 min with anti-CD 16 (60 µL), anti-CD 14 (20 µL) and anti-CD 3 (20 µL) immunomagnetic beads (MACS, CA, USA). These cells were passed through a magnetic column and the eluant contained an eosinophil population of >97 % purity. Eosinophil counts were performed using the Kimura staining technique [22].

B) Cell culture:

Eosinophils were resuspended in RPMI-1640 (2×10^6 cells/mL) and incubated at 37° C in the presence or absence of 100 ng/mL TNF-α. In pilot experiments, we optimized the conditions for MMP-9 release from eosinophils following stimulation by TNF-α. A generally accepted range for TNF-α concentration is between 1-100 ng/mL. We have therefore tested five different concentrations of TNF-α (0, 0.1, 1, 10, 100 ng/mL). A dose of 100 ng/mL showed the highest increase in MMP-9 secretion, and this concentration was used throughout the current study. Similarly, time-course experiments indicated that 24 h incubation of eosinophils with or without TNF-α was optimal, since, especially in

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unstimulated cells. at earlier time points (0, 1, 8 h) MMP-9 mRNA expression and protein release was low. In order to study the regulation of MMP-9 synthesis and release, cells were incubated with either actinomycin D (10^{-6} M), cycloheximide (10^{-6} M), the protein kinase C inhibitor H7 (500 μM), the NFκB inhibitor N-CBZ-LEU-LEU-LEU-AL (10 μM) or a neutralizing anti-human TNF-α antibody (0.04 μg/mL) for 24 h in the presence or absence of TNF-α. Except for the anti-TNF-α antibody, which was purchased from R&D Systems (Minneapolis, USA), all reagents were obtained from Sigma, St. Louis, USA.

Since transcription and translation inhibitors are known to be cytotoxic, cell viability was assessed after 24 h incubation by using trypan blue dye exclusion and an Annexin V-FITC Apoptosis Detection kit (PharMingen, ON, Canada) to detect dead cells. In all experiments described, cell viability was >90 % at all time points examined. These findings exclude the possibility that the observed increase in MMP-9 activity was caused by unspecific protein leakage due to cell death.

C) RT-PCR:

Total RNA was isolated from 2 x 10^6 eosinophils using the Quiagen RNeasy kit (Ontario, Canada). The average amount of RNA obtained from 2 x 10^6 eosinophils was 300 ng. One third of the RNA was reverse transcribed using superscript II reverse transcriptase (Gibco BRL, Ontario, Canada) and random hexamers (50A_{260} units) (Boehringer Mannheim GmbH, Mannheim, Germany) as primers. Thereafter, PCR was performed in 20 μL reactions using the following primers (25 μM) for MMP-9 (5'-CGTGGAGAGTCGAAATCTCTG-3'; 5'-CCAAAC TGGATGACGATGTCT-3'), TIMP-1 (5'-GGGGACACCAGAAGTCAACCAGA-3'; 5'-CTTTT CAGAGCCTTGGAGGAGCT-3') and TIMP-2 (5'-GATGCACATCACCCTCTGTG-3'; 5'-CTC GATGTCGAGAAACTCCTG-3'). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5'-C CACCCATGGCAAATTCCATGGCA-3'; 5'-TCAAGACGGCAGGTCAGGTCCACC-3') was used as a housekeeping gene internal standard. One tenth of the cDNA was used in PCR experiments. DNA amplification was

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obtained by annealing at 54° C for MMP-9 and TIMP-2, 50° C for TIMP-1 and 67° C for GAPDH for 30 sec, followed by an elongation step at 72° C for 1 min. All sequences were amplified over 30 cycles, except for TIMP-1 sequences, which needed 40 amplification cycles to be detected. The amplified fragment size for GAPDH, MMP-9, TIMP-1 and TIMP-2 was 598 bp, 331 bp 400 bp and 292 bp, respectively.

D) Gelatin zymography:

SDS-PAGE gelatin zymography (7 % polyacrylamide gel containing 2 % gelatin) was performed as follows [23,24]. Serum-free culture supernatants were assayed in order to study MMP-9 release, whereas intracellular MMP-9 protein was studied in lysed cell pellets. Supernatants were concentrated 5 x in Centricon 10 concentrators (Amicon, MA, USA). Cell pellets were lysed by 3 x 1 min freeze-thaw cycles on dry ice and a 37° C waterbath. The resulting homogenate was resuspended in RPMI-1640 in a volume equal to that of the supernatants. Phorbol-12-myristate-13-acetate (PMA)-treated (100 nM, 48 h at 37° C) HT-1080 fibroblast supernatants were used as a positive control for gelatinolytic activity and to distinguish MMP-9 activity from that of MMP-2. Following electrophoresis at 4° C, the gels were washed 3 x in 2.5 % Triton X-100 at room temperature. The gels were then incubated in 50 mM Tris-HCl buffer (pH 7.6) supplemented with 0.15 M NaCl, 5 mM $CaCl_2$ and 0.05 % NaN_3 at 37° C for 24 h. After incubation, the gels were stained for 1 h with 0.05 % Coomassie Brilliant Blue G-250. Thereafter, the gels were destained overnight in 20 % isopropanol/10 % acetic acid. All reagents were obtained from Sigma (ON, Canada) and the electrophoresis unit (Mini-Protean II Gel) was obtained from Bio-Rad (CA, USA). Proteolytic activity was identified as clear bands on a blue background.

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E) SDS-polyacrylamide gel electrophoresis and Western blotting :

Human eosinophils (2 x 10^6 cells/mL) were incubated with or without TNF-α (100 ng/mL) in RPMI-1640 for 24 h at 37° C. After 5 min centrifugation at 100 g supernatants were concentrated (10 x) and the cells were lysed by repeated freeze-thaw cycles (3 x 1 min) in 100 μL homogenisation buffer containing 0.5 mM EDTA (pH=7.4), 10 mg/mL PMSF, and a mixture of 5 mg/mL TAME, aprotinin and leupeptin. Untreated HT-1080 fibroblast supernatant was concentrated (20 x) and used as a positive control for TIMP-2. PMA-treated (100 nM, 48 h, 37°C) HT-1080 fibroblast supernatant (10 x concentrated) was used as a positive control for TIMP-1. Equal concentrations of eosinophil supernatant and cell homogenates (80-100 μg protein) were loaded onto a 12 % SDS-polyacrylamide gel and transferred electrophoretically (25 V, 35 min) onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) using the Semi-Dry Trans Blot System according to the manufacturer's instructions. The membrane was then blocked overnight in 5 % milk powder (Bio-Rad) in tris buffered saline (TBS) containing 0.02 % Tween at 4° C. In order to avoid non-specific background staining due to high concentrations of cationic proteins contained in the eosinophil preparations, the membrane was then blocked in 5 % milk powder in TBS containing 5 % goat serum (Gibco, ON, Canada) for 1 h. Thereafter, the membrane was probed with a mouse monoclonal anti-human TIMP-1 (1 μg/mL) or anti-human TIMP-2 antibody (5 μg/mL) in 5 % milk powder containing 5 % goat serum for 1 h at room temperature. Both antibodies were obtained from Cedarlane Laboratories Ltd (ON, Canada). The membrane was then incubated with a peroxidase-conjugated polyclonal anti-mouse antibody (1:5000) (Amersham, ON, Canada). Protein bands were detected by enhanced chemoluminescence (Amersham, ON, Canada).

F) Statistical analysis and optical density measurements:

Optical densities of PCR gels and of zymograms were measured using the Sigma Gel Analysis program (Jandel Scientific, version 1.0.5.0, 1995, USA). Band intensities of

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PCR products were expressed as percentage of a GAPDH standard ± SEM. In pilot experiments GAPDH expression was shown to be stable upon stimulation with IL-3, IL-5 and TNF-α. Band intensities of zymograms were expressed as percentage of treated vs. untreated cells ± SEM.

Densitometry is a quick and very convenient method to compare different band intensities on RT-PCR or gelatin-zymography gels. Advantages of this computerized technique are high sensitivity and rapidity in directly measuring large numbers of band intensities. Potential disadvantages include the possible subjective interpretations of band intensities during the measuring process, especially if performed by different investigators. In order to avoid these possible pitfalls in this study, only one investigator performed optical density measurements. We were, however, aware of the fact that this method is neither helpful in detecting absolute copy numbers or protein concentrations, nor able to detect subtle differences in gene expression or protein production.

For statistical analysis the unpaired student t-test was used (InStat, Graph Pad Software Inc., USA). A value of $p < 0.05$ was considered significant.

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RESULTS

1. MMP-9 and TIMP expression in human eosinophils:

Transcriptional expression of MMP-9 as well as TIMP-1 and TIMP-2 was studied in human peripheral blood eosinophils using RT-PCR. MMP-9 and TIMP-2 gene expression could be detected in both resting and TNF-α stimulated eosinophils (Fig. 2.1A), whereas TIMP-1 gene expression appeared to vary between different subjects. Stimulation of eosinophils for 24 h with TNF-α resulted in upregulation of MMP-9 gene expression (Fig. 2.1B) (29.5±4.5 and 40.5±3.5, respectively), although the increase did not achieve statistical significance ($p=0.09$, $n=4$). In contrast, TIMP-2 gene expression (Fig. 2.1B) in eosinophils appeared to decrease after TNF-α treatment (46.8±5 and 32.6±5, respectively), but the difference did again not achieve statistical significance ($p=0.08$, $n=5$). Throughout these preparations, GAPDH was equally and consistently expressed in unstimulated as well as in TNF-α stimulated eosinophils.

2. TNF-α increases MMP-9 activity in human eosinophils:

We examined the potential of TNF-α to increase MMP-9 release from human eosinophils using gelatin zymography (Fig. 2.2A). Addition of TNF-α (100 ng/mL) to human peripheral blood eosinophils significantly increased MMP-9 activity after 24 h incubation by 95±6 % ($p<0.01$, $n=13$) compared to untreated eosinophils (Fig. 2.2B). In order to ensure that this effect was specific to TNF-α and not due to some non-specific protein interactions, a neutralizing anti-TNF-α antibody was added to the cell cultures for 24 h. In the presence of TNF-α and anti-TNF-α antibody, MMP-9 activity remained at baseline values ($n=3$) (Fig. 2.2). Other cytokines, including IL-3 and IL-5, caused only a weak increase in MMP-9 activity.

We also studied the effect of TNF-α on the ratio of released versus intracellularly stored MMP-9. Therefore, supernatants and cell lysates of both resting and stimulated cells were analyzed in gelatin zymography ($n=4$) after a 24 h incubation period

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(Fig. 2.3A). A clear shift of MMP-9 activity from the lysed cell pellet into the supernatant could be observed upon TNF-α stimulation (Fig. 2.3B). In resting eosinophils MMP-9 activity could be detected in both, cell lysates (34 %) and supernatants (66 %). In TNF-α treated eosinophils, however, most of the MMP-9 protein (92 %) was released into the supernatant ($p<0.01$), with only 8 % retained in the cells ($p<0.01$).

3. The increase in MMP-9 activity is regulated at different levels:

In order to gain insights into MMP-9 activation, eosinophils were incubated with TNF-α in the presence or absence of transcription and translation inhibitors, as well as with a protein kinase C (PKC) inhibitor. In RT-PCR experiments, the combination of TNF-α with transcription and translation inhibitors, as well as the PKC inhibitor H7, decreased MMP-9 mRNA levels compared to TNF-α-stimulated cells (Fig.2.4A). These results were also confirmed at protein level (Fig. 2.4B). The transcription inhibitor actinomycin D decreased MMP-9 activity by 61 ± 7 % ($p<0.01$, $n=7$) compared to TNF-α treated cells. To investigate whether or not the nuclear factor κB (NFκB) was involved in TNF-α-induced upregulation of MMP-9 transcription, an NFκB inhibitor, N-CBZ-LEU-LEU-LEU-AL, was used. Inhibition of NFκB resulted in a significant reduction of MMP-9 gelatinolytic activity by 61 ± 12 % ($p<0.01$, $n=5$) compared to TNF-α-stimulated eosinophils. These results indicate that upon TNF-α stimulation MMP-9 protein was newly synthesized. In addition, we found that the TNF-α-stimulated MMP-9 activity profile was also regulated at translational level. Eosinophils were incubated with TNF-α in the presence or absence of the protein synthesis inhibitor cycloheximide. The latter decreased TNF-α-induced MMP-9 activity by 60 ± 11 % ($p<0.01$, $n=7$) compared to TNF-α treated cells. To determine whether PKC was involved in the TNF-α-induced effects, eosinophils were incubated in the presence of this cytokine with or without the PKC inhibitor, H7. Addition of H7 to TNF-α stimulated eosinophils resulted in inhibition of MMP-9 activity by 57 ± 5 % ($p<0.01$, $n=8$) compared to TNF-α stimulated cells. The presence of these inhibitors alone did not affect constitutive MMP-9 secretion using gelatin zymography ($n=3$) (Fig. 2.4B).

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4. TIMP protein expression in human eosinophils:

We investigated the ability of human eosinophils to produce TIMP-1 and TIMP-2 proteins using Western blotting. Eosinophil supernatants and cell lysates were assayed in order to detect released and intracellular TIMP proteins. We detected TIMP-1 protein in only two out of four subjects studied. The pattern of TIMP-1 release from cells of these two subjects also appeared to vary between different individuals. In one subject, TIMP-1 localized primarily intracellularly, whereas in the other the majority of TIMP-1 protein was secreted. In both cases, TNF-α stimulation increased the levels of TIMP-1 protein compared to resting cells. In the other two subjects, no TIMP-1 protein could be detected in either supernatant or cell lysate.

We further investigated the ability of human eosinophils to produce TIMP-2. Eosinophils obtained from four subjects showed TIMP-2 protein expression (Fig 2.5). However, like TIMP-1, the pattern of protein release was inconsistent and the amounts of protein varied among subjects, with only two of the four subjects showing similar intracellular levels of TIMP-2. In both cases, TIMP-2 protein was released at an increased rate following TNF-α stimulation (Fig. 2.5). Eosinophils from the other two subjects showed TIMP-2 protein exclusively intracellularly.

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DISCUSSION:

The recent description of the capacity of eosinophils to release MMP-9 has opened new vistas towards our understanding of the process of airway remodeling in asthma [25]. Our novel data revealed that human eosinophil-derived MMP-9 expression and secretion is upregulated upon TNF-α stimulation. Gelatin zymography provided an excellent method to show MMP-9 activity in these cells. NFκB was previously shown to be involved in MMP-9 activation in fibroblasts [26], transformed cells [27;28] and renal cells [29]. Our findings also confirm the validity of the hypothesis that TNF-α-induced binding of NFκB to the MMP-9 promoter is a requirement for transcription activation [26]. However, other transcriptional factors such as activator protein AP-1 are also likely to be involved [28]. Our data shed new important light on the possible involvement of PKC in MMP-9 transcription and translation. However, this aspect requires further investigation. Studies in uterine fibroblasts have suggested that MMP-9 production is PKC-independent [16]. In addition, PMA, a PKC activator, increased MMP-9 secretion in alveolar macrophages of healthy controls, but not asthmatic subjects [30]. However, PKC appears to be one of the intracellular signaling mediators of TNF-α effects. In bronchial epithelial cells, TNF-α has been shown to increase PKC activity by 3-5 fold [31]. Our data underscore the importance of PKC in MMP-9 expression and secretion and confirm this in eosinophils.

TNF-α is only one out of many cytokines released at the inflammatory site. In preliminary experiments we investigated the effects of two other important cytokines in eosinophilic inflammation, namely IL-3 and IL-5 [32;33]. However, both cytokines were able to elicit only a weak increase in MMP-9 activity. Similar results have been reported by Fujisawa et al., who could not induce a significant increase in MMP-9 release from eosinophils upon IL-5 stimulation [34]. These data emphasize the potential of TNF-α as a major regulatory molecule for MMP-9 regulation in eosinophils. We can not, however, exclude the possibility that *in vivo* interactions between different combinations and concentrations of cytokines may have additional synergistic and/or antagonistic effects on MMP-9 regulation.

In addition to different types and concentration of cytokines present at the inflammatory site, the equilibrium between MMPs and their specific tissue inhibitors

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(TIMPs) may also determine the degree of tissue degradation. It was, therefore, tempting to speculate that the production and secretion of both MMP-9 and TIMPs might be altered in an inflammatory environment. Other investigators who described TIMP-1 as an inducible protein in contrast to TIMP-2 [8], have reported similar observations. The inconsistent pattern of release, however, puts into question the physiologic relevance of TIMP production by eosinophils. More importantly, other inflammatory and tissue structural cells in the airways, including monocytes/macrophages [8], epithelial cells [35], and myofibroblasts [36] are known to produce considerable amounts of TIMP proteins. It is, therefore, likely that, in addition to limited autocrine TIMP production, eosinophil MMP-9 activity is primarily regulated by TIMP proteins secreted from other cells at the inflammatory site.

Increased production of MMP-9 at the inflammatory site has been shown to be associated with airway tissue remodeling [37]. We suggest that in a TNF-α-rich inflammatory milieu MMP-9 activity might be increased. This does not exclude the possibility that other MMPs, such as the gelatinase MMP-2, together with collagenases and stromelysins, may participate in increased interstitial matrix turnover in asthmatic airways [38]. The magnitude of contribution of these other MMPs, in addition to MMP-9, to overall tissue destruction in hyperreactive airways, remains to be elucidated.

Our study relied on peripheral blood eosinophils obtained from atopic asthmatic subjects. It would be ideal to compare these observations with cells obtained from broncho-alveolar lavage [39], segmental bronchoprovocation [40], mucosal biopsies [41] or induced sputum samples [42] from asthmatic patients. There remains a paucity of data on possible differences between blood and tissue eosinophils. However, it is now generally accepted that peripheral blood eosinophils are a useful model of their tissue counterparts. Eosinophil numbers in the peripheral blood of asthmatics appear to correlate significantly with tissue cell counts [42] and the degree of airflow obstruction [43].

In conclusion, our data propose a major role for TNF-α in MMP-9 regulation in asthmatic airways and an important function for the eosinophil in interstitial matrix turnover and airway tissue remodeling. These findings may have significant implications for future anti-eosinophil therapies in asthma and related conditions.

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Figure 2.1

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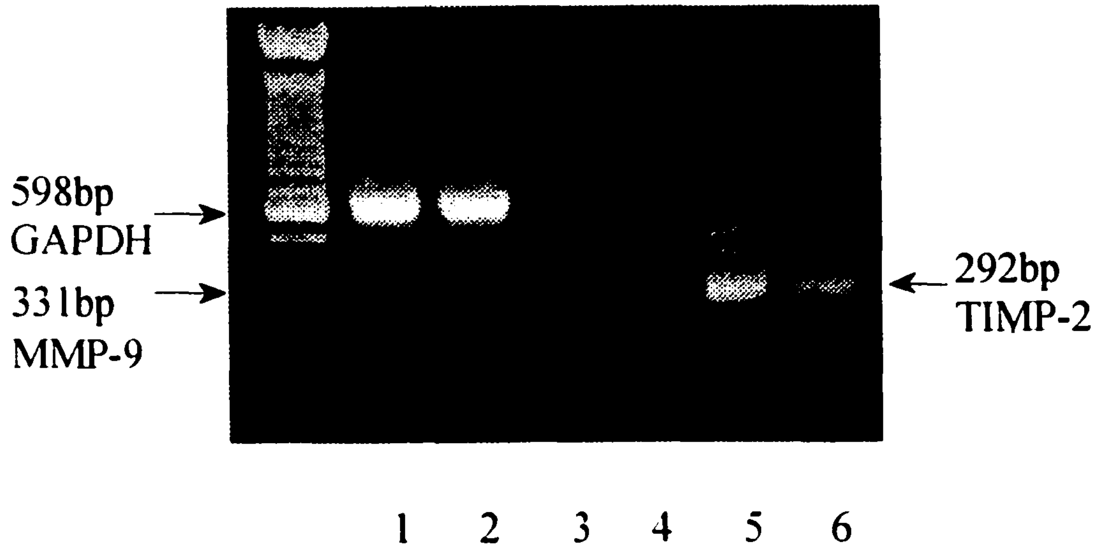

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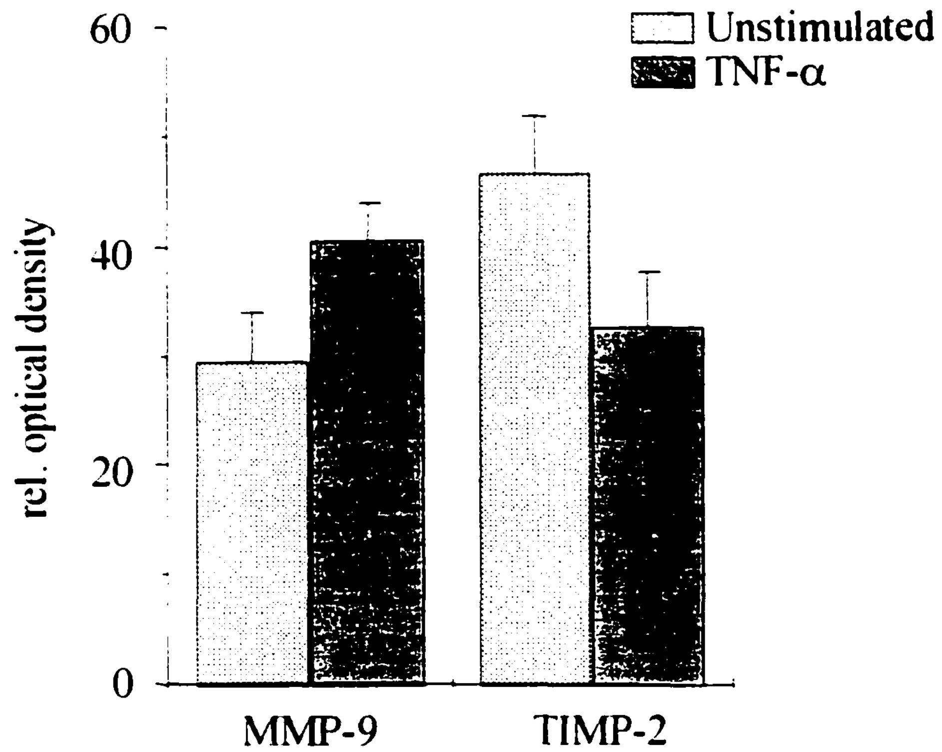

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Figure 2.2

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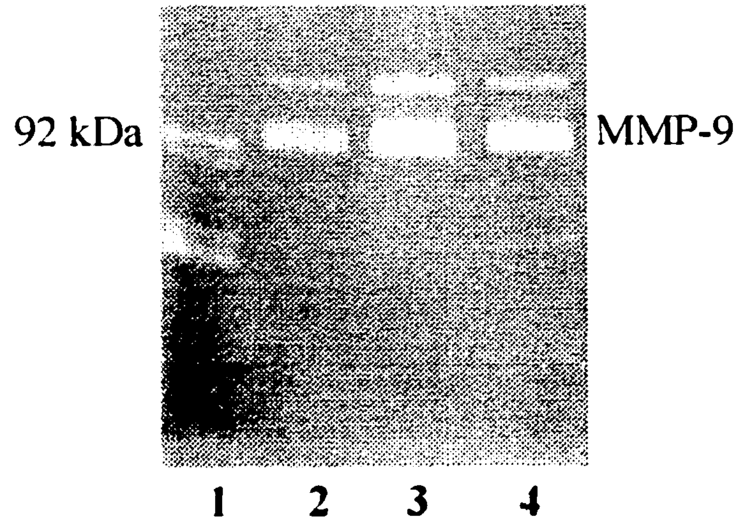

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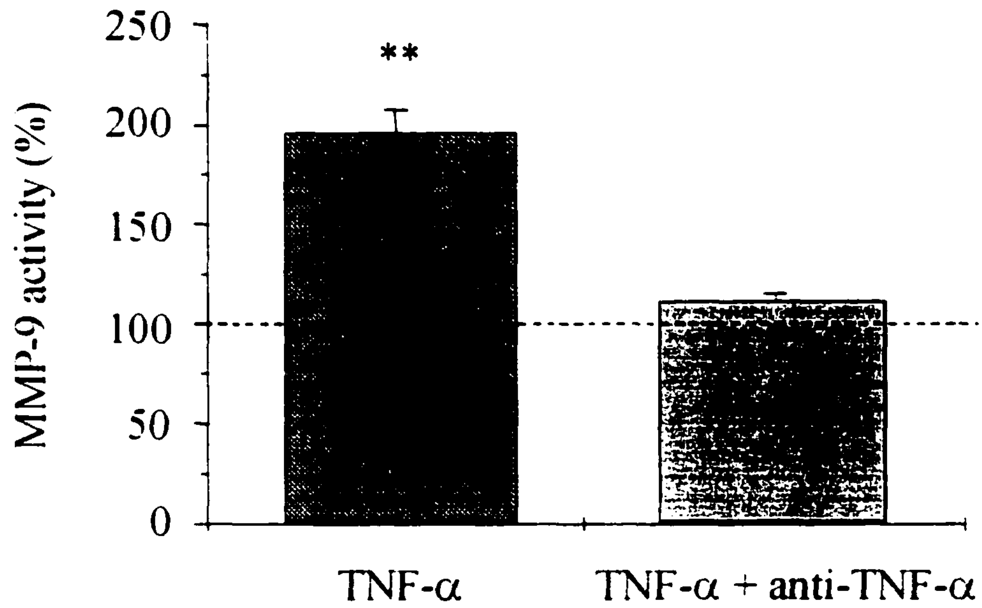

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Figure 2.3

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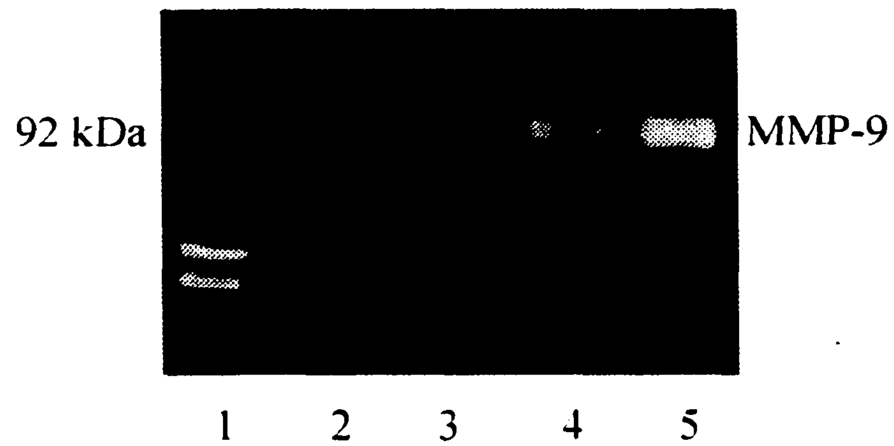

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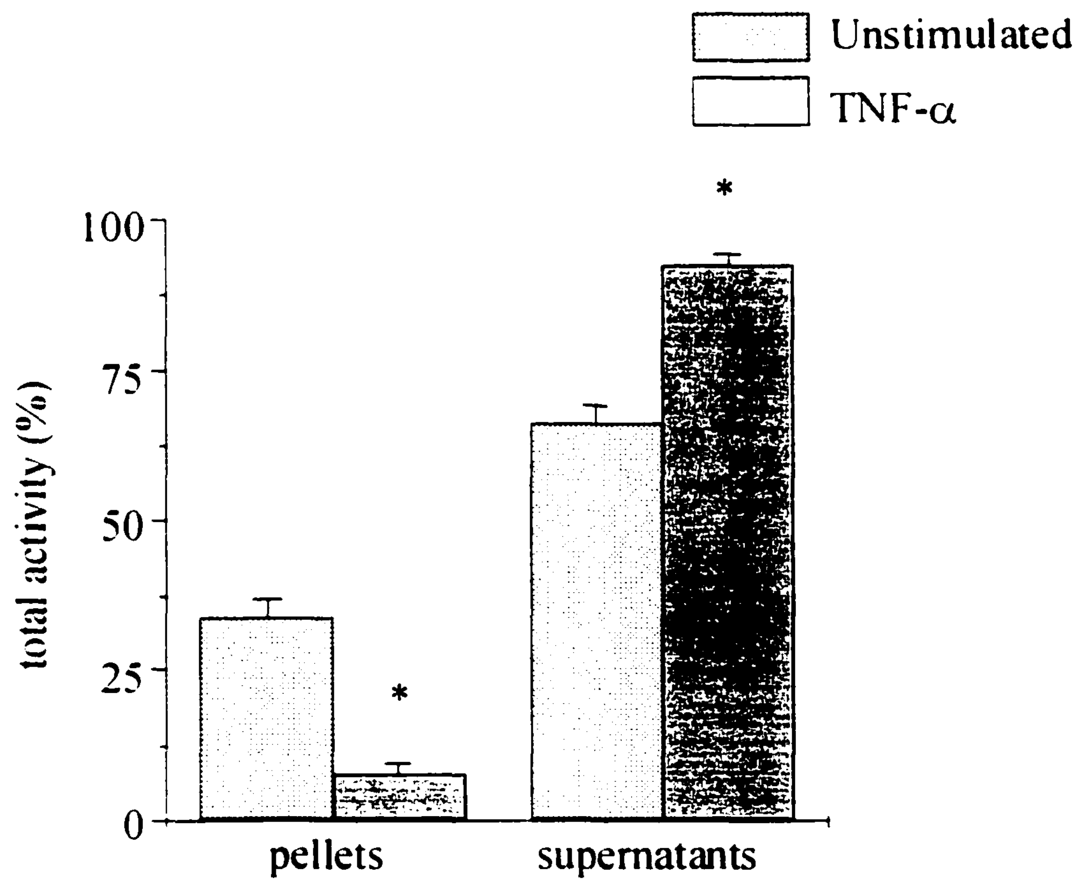

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Figure 2.4

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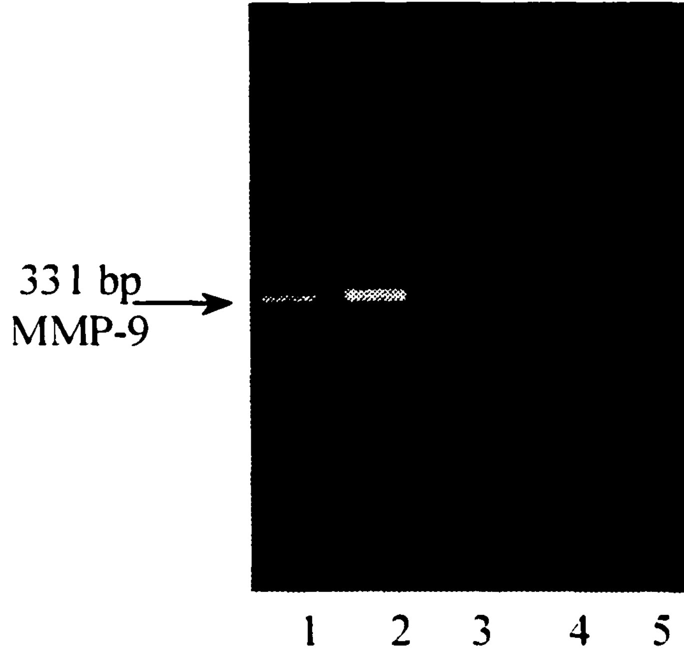

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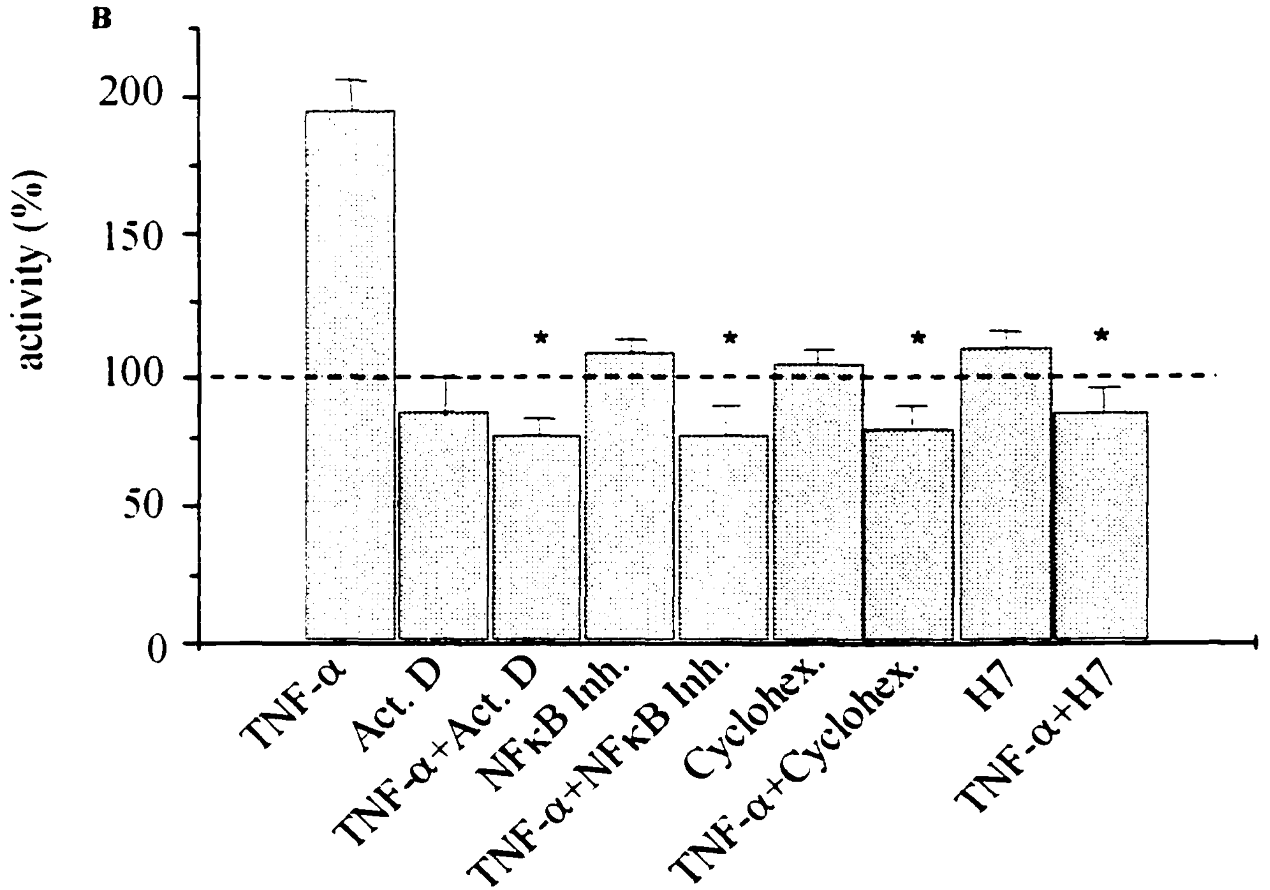

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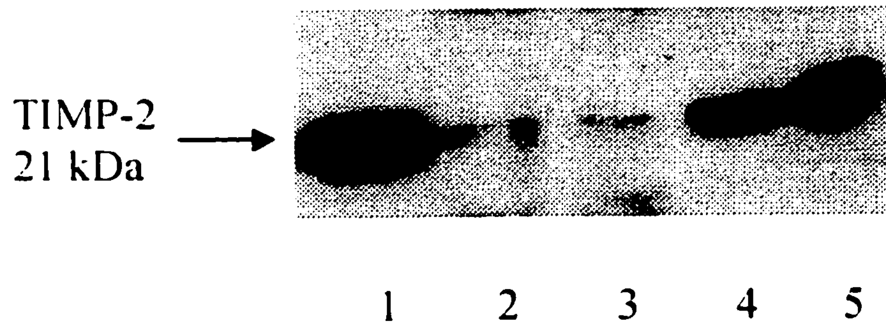
Figure 2.5



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LEGENDS TO FIGURES

Fig.2. 1

Panel A shows a representative RT-PCR experiment of resting and TNF-α stimulated eosinophils. Cells were incubated for 24 h at 37° C in the presence or absence of TNF-α (100 ng/mL). Sequences for GAPDH (lane 1 and 2), MMP-9 (lane 3 and 4) and TIMP-2 (lane 5 and 6) were amplified over 30 cycles. MMP-9 expression in eosinophils was increased upon TNF-α stimulation (lane 4) compared to unstimulated cells (lane 3). In contrast, TIMP-2 expression was weaker in TNF-α treated (lane 6) than in untreated cells (lane 5). No difference could be observed in the expression of GAPDH between untreated (lane 1) and TNF-α treated (lane 2) eosinophils.

Panel B shows densitometric analysis data of MMP-9 and TIMP-2 expression in unstimulated and TNF-α stimulated eosinophils using RT-PCR. Cells (2 x 10^6) were incubated for 24 h in the presence or absence of TNF-α. MMP-9 and TIMP-2 band intensities are expressed as percentages (mean ± SEM) of a GAPDH internal standard. After TNF-α stimulation MMP-9 expression increased by 11 % compared to unstimulated eosinophils. In contrast, TIMP-2 expression decreased by 14 % upon TNF-α stimulation.

Fig.2. 2

Panel A shows MMP-9 activity of unstimulated and TNF-α (100 ng/mL) stimulated eosinophil supernatants using gelatin zymography. After a 24 h incubation period supernatants obtained from 2 x 10^6 cells were 10 x concentrated and analyzed by gelatin zymography. PMA-stimulated HT-1080 fibroblast supernatant (lane 1) was used as a positive control for active MMP-9 (MW: 92 kDa). Unstimulated eosinophils constituitively showed MMP-9 gelatinolytic activity (lane 2). Upon TNF-α stimulation MMP-9 gelatinolytic activity was significantly increased (lane 3). The TNF-α specificity of this effect was shown using a neutralizing anti-TNF-α antibody, which clearly decreased MMP-9 activity (lane 4).

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Panel B shows densitometric analysis of cell-free eosinophil supernatants using gelatin zymography. Eosinophils were incubated for 24 h with or without TNF-α, or TNF-α and anti-TNF-α. Results are expressed as percentage of activity of TNF-α treated to resting cells (mean ± SEM). MMP-9 gelatinolytic activity of resting cells is expressed as 100 %. TNF-α significantly increased MMP-9 gelatin-degrading activity by 95 % ($p<0.01$) compared to unstimulated cells. This effect could be reversed by the addition of neutralizing TNF-α antibody.

Fig.2. 3

Panel A shows intracellular and secreted MMP-9 protein of resting and TNF-α (100 ng/mL) stimulated eosinophils after 24 h incubation in a representative gelatin zymography experiment. PMA-stimulated HT-1080 fibroblasts (lane 1) were used as a positive control for active MMP-9 (MW: 92 kDa). Resting eosinophils constituitively showed MMP-9 gelatinolytic activity in both, lysed cell pellet (lane 2) and supernatant (lane 4). After TNF-α stimulation the pellet was almost completely depleted of MMP-9 (lane 3) and the majority of active MMP-9 protein was released into the supernatant (lane 5)

Panel B shows densitometric analysis of intracellular and released MMP-9 in lysed eosinophil pellets and supernatants using gelatin zymography. Results are expressed as percentage of total MMP-9 activity (mean ± SEM). After 24 h incubation resting eosinophils showed constituitively MMP-9 activity in pellets (39 %) as well as supernatants (61 %). Upon TNF-α stimulation, MMP-9 activity in the pellets decreased (8 %), whereas active MMP-9 levels were increased in the supernatants (91 %).

Fig.2. 4

Panel A shows the regulation of TNF-α-induced increase in MMP-9 expression using RT-PCR. Eosinophils were incubated for 24 h in the presence or absence of TNF-α and with actinomycin D, the NFκB inhibitor N-CBZ-LEU-LEU-LEU-AL, or the protein kinase C inhibitor H7. Resting eosinophils showed constitutively MMP-9 (product size 331 bp) expression (lane 1). Upon TNF-α stimulation MMP-9 expression was markedly

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upregulated (lane 2). The addition of actinomycin D (lane 3), the NFκB inhibitor (lane 4) and H7 (lane 5) to TNF-α stimulated cells clearly decreased MMP-9 expression.

Panel B shows densitometric analyses of MMP-9 regulation in gelatin zymography. Eosinophils were incubated with or without TNF-α and actinomycin D, the NFκB inhibitor N-CBZ-LEU-LEU-LEU-AL, cycloheximide, or the protein kinase C inhibitor H7. Results are expressed as percentage of activity of TNF-α treated to untreated cells (mean ± SEM). MMP-9 gelatinolytic activity of untreated cells is expressed as 100 %. TNF-α significantly upregulated MMP-9 activity by 95±6 %. The combination of TNF-α and actinomycin D or the NFκB inhibitor decreased MMP-9 activity significantly by 61±7 % and 61±12 %, respectively, compared to TNF-α stimulated cells. Similarly, cycloheximide decreased MMP-9 activity by 60±11 % and H7 by 57±5 % compared to TNF-α treated cells. The inhibitors alone did not affect constituitive MMP-9 release. In all experiments the p value was <0.01.

Fig.2. 5

Figure 2.5 shows a Western blot experiment for TIMP-2. As a positive control for TIMP-2 unstimulated HT-1080 fibroblast supernatant was used (lane 1). Lane 2 and 3 show similar expression of TIMP-2 protein in cell pellets of resting (lane 2) and TNF-α stimulated (100 ng/mL) eosinophils (lane 3) from this patient. In the supernatant increased amounts of released TIMP-2 protein were found after TNF-α stimulation (lane 5) compared to the supernatant of resting cells (lane 4).

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CHAPTER 3

The involvement of ion channels in human eosinophil respiratory burst

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INTRODUCTION

During respiratory burst, eosinophils produce reactive oxygen species such as superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). These mediators have been implicated in bronchial tissue damage and persistent inflammation in the airways [1]. The importance of $O_2^{\bullet-}$ as a major damaging molecule in asthmatic airways is suggested by the fact that several anti-asthma drugs have been shown to inhibit $O_2^{\bullet-}$ release from human eosinophils [2].

There is increasing evidence to suggest that ion currents play an important role in eosinophil activation. The H^+ channels serve to extrude H^+ ions generated during eosinophil respiratory burst [3;4]. However, in addition to H^+ channels, there is also significant evidence that other ion channels might be involved in eosinophil respiratory burst. For example, K^+ channel blockers have been shown to inhibit $O_2^{\bullet-}$ release from eosinophils [5]. Similarly, nedocromil sodium, an anti-asthma drug, was shown to block Cl^- flux [6]. Other studies have demonstrated that activation of Ca^{2+} channels is required for eosinophil mediator release [7]. All these studies suggest that in addition to H^+ channels, control of ion movement by other channels may be an important factor in eosinophil activation.

The aim of the present study was to explore the role of Cl^- channels in $O_2^{\bullet-}$ production in human eosinophils. We have used PMA to stimulate $O_2^{\bullet-}$ production by eosinophils. This agent is recognized as a potent activator of eosinophil respiratory burst, and its mechanism of action has been extensively investigated [8]. Our data show, for the first time, that activation of Cl^- channels is an integral event associated with respiratory burst activity in human eosinophils.

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MATERIALS AND METHODS

A) Measurements of $O_2^{\bullet-}$ production in purified human eosinophils:

Peripheral blood eosinophils were purified from atopic asthmatic volunteers who had given their informed consent, as described previously in Chapter 2 [9;10]. Superoxide generation was determined by SOD-inhibitable reduction of cytochrome *c*. Eosinophils (20×10^6 cells/mL) were resuspended either in a high-Cl^- buffer (mM: 137 NaCl, 6.4 KCl, 4.3 Na_2HPO_4, 1.4 KH_2PO_4, 1.2 $MgCl_2$, 0.5 $CaCl_2$, 5 glucose), or a low-Cl^- buffer (mM: 127 Na-gluconate, 10 NaCl, 6.4 K-gluconate, 4.3 Na_2HPO_4, 1.4 KH_2PO_4, 1.2 $MgCl_2$, 0.5 Ca^{2+} gluconate and 5 glucose). In experiments with K^+ channel blockers, eosinophils were resuspended in a high-K^+ buffer (mM: 5 NaCl, 140 M KCl, 4.3 Na_2HPO_4, 1.4 KH_2PO_4, 1.2 $MgCl_2$, 0.5 $CaCl_2$, 5 glucose, 10 HEPES). All buffers were supplemented with 0.1 % BSA. The cells were kept in the above buffers for 1 hour before starting the experiments. Eosinophils (1×10^6/mL) and cytochrome *c* (114 μM, Sigma) were pre-incubated for 10 min in the presence or absence of clotrimazole, $BaCl_2$, amiloride, DPC, niflumic acid, acetozolamide and diltiazem (all from Sigma), or DIDS and DNDS (Molecular Probes). The effect of these chemicals on PMA-stimulated $O_2^{\bullet-}$ production was measured in a spectrophotometer (Becton Dickinson) at a wavelength of 550 nm, for up to 20 min. To determine the effects of halides on $O_2^{\bullet-}$ production, Cl^- in the buffer was replaced by Br^-, I^-, or F^- in equimolar concentrations. In experiments using low-Cl^- buffer, NaCl was exchanged for Na-gluconate. The pH of all solutions was adjusted to 7.4. The amount of $O_2^{\bullet-}$ generated by eosinophils was expressed in nM/(min 10^6 cells) $\pm$ SEM using a molar extinction coefficient ε for cytochrome *c* of 2.11×10^4 M^{-1} cm^{-1}.

The dose-response curve for the stimulation of $O_2^{\bullet-}$ production (P) by PMA was fitted by

$$P = \frac{P_{max}}{\left(\frac{K_d}{C}\right)^n + 1} \qquad (1)$$

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where P_{max} is the maximum production of $O_2^{\bullet-}$, C is the concentration of PMA, K_d is the half maximal concentration for the reaction between PMA and its intracellular target, and n is the cooperativity factor.

B) Patch clamp experiments:

Whole cell recordings were obtained using the amphotericin B-perforated patch clamp technique. Patch pipettes were pulled from borosilicate glass (A-M Systems, USA) with use of a Narishige puller (Tokyo, Japan). The pipette tip was dipped into pipette solution (137 mM KCl, 6.4 mM NaCl, 4.3 mM $NaHPO_4$, 1.4 mM KH_2PO_4, 1.2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM EGTA, 4 mM glucose and 0.1 % BSA) and the pipette was then back-filled with the same solution containing amphotericin B (240 µg/mL, Sigma). Pipette resistances were between 3-8 MΩ and recordings were obtained using a patch clamp amplifier (EPC-7, List Medical, Germany) in the voltage clamp mode. The holding potential was -60 mV, and 20 mV steps ranging from -80 to +80 mV, were applied every 200 ms. The contribution of Cl^- channels to the whole-cell current was evaluated using high- and low-Cl^- buffers. When cells were bathed in the low-Cl^- buffer, 100 mM Cl^- in the pipette was replaced by gluconate. The contribution of K^+ channels to the whole-cell current was evaluated by replacing K^+ in the bath and the pipette buffers by Cs^+. Data were analyzed using custom-written patch clamp software (kindly provided by Dr. A.S. French, Dalhousie University). Analysis of whole cell currents was performed at +80 mV. Liquid junction potentials, which develop whenever solutions with two different compositions come into contact, were calculated as previously described[11]. All experiments were performed at room temperature.

C) RT-PCR:

Total RNA was isolated from $2x10^6$ eosinophils using the Qiagen RNeasy Mini kit (Qiagen). The average amount of RNA obtained from $2x10^6$ eosinophils was 300 ng. One third of the RNA was reverse transcribed using superscript II reverse transcriptase (Gibco) and random hexamers ($50A_{260}$ units, Boehringer Mannheim) as primers.

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Thereafter, PCR was performed in 20 μL reactions. The expression of CLC-2, CLC-3, CLC-4, CLC-5, CLC-6 and CFTR in eosinophils was studied using the primer pairs described in Table 3.1. As a positive control for CFTR and CLC gene expression, mRNA was isolated from the human airway submucosal cell line Calu-3 and the human embryonic kidney cell line HEK 293, respectively. One tenth of the cDNA was used in PCR experiments. DNA amplification was obtained by annealing for 30 sec at 62°C for CLC-2, 60°C for CLC-3 and CLC-4, 57°C for CLC-5, 60°C for CLC-6, and 55°C for CFTR, respectively. This was followed by an elongation step at 72 °C for 1 min. DNA sequences were amplified during 40 cycles. The sizes of the expected amplified products are shown in Table 3.1.

D) Statistical Analysis:

Data are presented as means ± SEM; n refers to the number of experiments. The unpaired Student's t-test was used to compare the means of two groups. Statistical differences among the means of multiple groups were determined using one-way analysis of variance (ANOVA). A value of $p<0.05$ was considered statistically significant.

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RESULTS

1) Production of $O_2^{\bullet-}$ by human eosinophils:

Eosinophils stimulated with PMA released $O_2^{\bullet-}$ in a dose- and time-dependent manner (Fig. 3.1). At doses of 1 ng/mL PMA, no substantial $O_2^{\bullet-}$ generation could be detected within 15 min. At doses higher than 10 ng/mL PMA, $O_2^{\bullet-}$ production reached a plateau between 8-10 min after stimulation (Fig. 3.1A). Figure 3.1B shows the release of $O_2^{\bullet-}$ (nM/min 10^6 cells) as a function of the PMA concentration. The steepness of the dose-response curve is reflected in the relatively high cooperativity value (3.5) obtained by fitting equation 1 to the data (see Materials and Methods). The half-maximal concentration of PMA for stimulation of $O_2^{\bullet-}$ production in human eosinophils was 4.98 ng/mL. In the remaining experiments, 10 ng/mL PMA was used, since this dose evoked a consistent response in eosinophils obtained from different individuals. Unstimulated eosinophils from all donors tested did not show any detectable $O_2^{\bullet-}$ production.

As reduction of cytochrome *c* is sensitive to other oxygen- or nitrogen-derived free radicals, SOD was added to PMA-stimulated eosinophils (Fig. 3.1A). In the presence of SOD, the $O_2^{\bullet-}$ production was reduced by more than 96 % ($n=3$, $p<0.01$). These results suggested that the majority of free radicals produced by eosinophils upon PMA-stimulation were $O_2^{\bullet-}$ anions.

2) The role of ion channels in eosinophil $O_2^{\bullet-}$ production:

Figure 3.2 shows the effects of different channel blockers on $O_2^{\bullet-}$ generation in eosinophils. In preliminary experiments we established that each of these drugs alone had no effect on $O_2^{\bullet-}$ production in unstimulated eosinophils. Clotrimazole, a blocker of intermediate and large conductance Ca^{2+}-activated K^+ channels [12], had no effect on $O_2^{\bullet-}$ production ($n=7$). Another K^+ channel blocker, barium, reduced $O_2^{\bullet-}$ generation by 18 ± 6 % ($n=4$, $p<0.05$), suggesting the involvement of clotrimazole-insensitive K^+

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channels in this process. Amiloride, an inhibitor of Na^+ channels and the Na^+/H^+ exchanger, had no effect on $O_2^{•-}$ generation (n=7). In the absence of DIDS, eosinophils produced 10±0.9 nM/(min 10^6 cells) of $O_2^{•-}$.

The role of Cl^- channels in PMA-induced $O_2^{•-}$ generation was studied using DIDS, niflumic acid, DPC and DNDS. DIDS inhibited $O_2^{•-}$ production in a dose-dependent manner (0-100 μM). Significant reduction of $O_2^{•-}$ production was observed at DIDS concentrations of 50 μM (31±7%, $p<0.01$, n=10) and 100 μM (67±18 %, $p<0.01$, n=3). Niflumic acid, an inhibitor of Ca^{2+}-sensitive Cl^- channels, and DPC, an inhibitor of CFTR Cl^- channels, had no effect on $O_2^{•-}$ generation (n=3 and 5, respectively). DNDS, an inhibitor of outwardly rectifying Cl^- channels [13], the Na^+-HCO_3^- cotransporter [14], and Cl^-/HCO_3^- exchanger [15], had no significant effect on $O_2^{•-}$ production (Fig. 3.2). This lack of involvement of the Cl^-/HCO_3^- exchanger in $O_2^{•-}$ generation was confirmed by RT-PCR and Western blot experiments. Both techniques were unable to detect mRNA or protein for any of the known three Cl^-/HCO_3^- exchanger isoforms (n=3). In addition, acetozolamide, an inhibitor of the carbonic anhydrase, had no effect on $O_2^{•-}$ generation, indicating that HCO_3^- ions were not involved in this process (Fig. 3.2). Diltiazem, a blocker of voltage-gated L-type Ca^{2+} channels, inhibited PMA-stimulated $O_2^{•-}$ generation by 35±8 % (n=3, $p<0.05$).

Anion channels are often characterized by determining their relative permeability to different halides. In this study, we replaced Cl^- ions in the high-Cl^- buffer with Br^-, I^- or F^-, in order to determine their effects on $O_2^{•-}$ production. Substitution of Cl^- ions with Br^- had no significant effect on $O_2^{•-}$ production (n=3, $p>0.05$). In the presence of I^- and F^-, $O_2^{•-}$ generation decreased by 31 % and 99 % (n=3, $p<0.01$), respectively, when compared with the Cl^- containing solution. The overall effects of halides on $O_2^{•-}$ production could be described by the sequence

$Cl^- \geq Br^- > I^-$. Complete inhibition of $O_2^{•-}$ production by another halide, F^-, is likely due to its interactions with cellular enzymes, particularly, G proteins [16].

Further involvement of Cl^- channels in $O_2^{•-}$ production was investigated in low- and high-Cl^- buffers (Fig. 3.3). In a high-Cl^- buffer, $O_2^{•-}$ production was 10.6±0.6 nM/(min 10^6 cells) and 7.4±0.9 nM/(min 10^6 cells) in the absence and presence of 50 μM DIDS (n=10, $p<0.05$), respectively. In contrast, in low-Cl^- buffer, $O_2^{•-}$

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production was 6.1±1 nM/(min 10^6 cells) and 6.05±1.2 nM/(min 10^6 cells), in the absence and presence of 50 μM DIDS (n=3), respectively. These experiments showed a requirement for extracellular Cl^- in eosinophil $O_2^{\bullet-}$ production.

3) Patch clamp studies:

We used the perforated patch clamp technique to characterize whole cell currents in human eosinophils. This modification of a conventional whole-cell recording is commonly used for measuring the electrical signal while maintaining the intracellular environment. Figures 3.4A,B show representative recordings in high- and low-Cl^- buffers, and the corresponding current-voltage relationships. Replacement of 134 mM Cl^- in the bath solution with gluconate, reduced the whole-cell current by 43±6 % (n=12, $p<0.01$), and caused a shift in the reversal potential from -27±3 mV to -9±2 mV ($p<0.01$, n=12), suggesting a significant contribution of Cl^- ions to the whole-cell current. This conclusion was further confirmed by showing that 50 μM DIDS reduced the whole cell current by 43±10 % (n=4, $p<0.01$) and caused a shift in the reversal potential (-35±6 to -16±8 mV; n=4, $p<0.01$).

The contribution of cation channels to the unstimulated whole-cell current was investigated using amiloride and cation substitution. Amiloride had no effect on the whole-cell current (n=4), indicating that amiloride-sensitive Na^+ channels did not significantly contribute to the whole-cell current. In contrast, replacement of K^+ with Cs^+ ions in the bath solution reduced the whole-cell current by 49 % (n=6, $p<0.01$), indicating an important contribution of K^+ channels to the unstimulated whole-cell current.

A summary of the effects of PMA treatment on the whole-cell current is shown in Fig. 3.4D. In high-Cl^- buffer, PMA increased whole cell currents by 168±23 % (n=4, $p<0.001$). In the presence of DIDS, the PMA effect was reduced to a 75±28 % increase over baseline current values (n=6, $p<0.05$), indicating that DIDS-sensitive Cl^- channels were affected by PMA treatment. In the low-Cl^- buffer, PMA increased the whole cell

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current by 62±18 % (n=7, $p<0.05$), which was significantly less than in the high-Cl^- buffer (n=7, $p<0.01$).

While K^+ channels were major contributors to the unstimulated whole-cell current, their function was not affected by PMA treatment. Equimolar replacement of K^+ with Cs^+ cations had no effect on the PMA-stimulated whole cell current (increase of 141±18 %, n=6, $p>0.05$). This indicates that the main target for PMA action is most likely DIDS-sensitive Cl^- channels.

4) Cl^- channel gene expression in eosinophils:

It has been shown before that CFTR Cl^- channels are present in human lymphocytes [17]. However, the expression of CFTR or any other Cl^- channel in human eosinophils has not been studied. Therefore, we have investigated gene expression of CFTR and some members of the CLC family in eosinophils using RT-PCR (Fig. 3.5). Our data show, that in contrast to lymphocytes, eosinophils do not express CFTR Cl^- channel mRNA. RT-PCR studies of ClC-2, -3, -4, -5 and -6 have only shown CLC-3 mRNA expression.

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DISCUSSION

The central observation of this report is that Cl^- channels play an important role in $O_2^{\bullet-}$ generation by PMA-stimulated eosinophils. The channel most likely involved in this process is ClC-3, a member of the ClC family. Superoxide ($O_2^{\bullet-}$) is generated as a result of a single electron transfer from cytosolic NADPH to external O_2, catalyzed by the NADPH oxidase complex [18]. This is an electrogenic process that causes depolarization of the plasma membrane, and an increase in the intracellular H^+ concentration [18]. In order to prevent massive membrane depolarization and a rapid fall in intracellular pH, it has been proposed that an efflux of H^+ ions through a H^+ channel provides the necessary charge compensation [19]. The experiments presented in this study show that other ion channels are also likely to be involved in this process. In particular, during the initial phase of $O_2^{\bullet-}$ production, a Cl^- influx may counteract the depolarization of the plasma membrane. When Cl^- is prevented from entering the cell, uncompensated depolarization of the eosinophil plasma membrane could result in impaired function of NADPH oxidase.

Patch clamp studies indicated that under unstimulated conditions, the whole-cell current in human eosinophils was dominated by Cl^- and K^+ conductances. This conclusion was based on the ion substitution studies and the use of ion channel blockers. Since ionic conductances control the membrane potential [20], it is likely that changes in transmembrane ion gradients affected the membrane potential, and thus, the activity of the NADPH oxidase.

The presence of H^+ currents in human eosinophils has been shown in several studies [21;22]. However, under our experimental conditions, a significant contribution of H^+ channels to the whole cell current was unlikely. H^+ currents show characteristic time-dependent activation at depolarizing voltages [23]. Since the single channel conductance of H^+ channels is very small (~10 fS) [23], studies of H^+ currents are usually performed at low pH (~5.5) to increase H^+ ion concentration and the current amplitude. The whole cell current recorded in our study did not show any significant time dependence. Furthermore, in our experiments, the pH of pipette and bath solutions was adjusted to 7.4, corresponding to a H^+ concentration of less that 40 nM. Under these conditions it is

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unlikely that H^+ ions could contribute more to the whole-cell current than 145 mM of K^+ or Cl^- ions.

The addition of PMA leads to the activation of the NADPH oxidase and to the phosphorylation of a wide range of proteins, including different types of ion channels [24]. In eosinophils, application of PMA significantly activated Cl^- currents, as shown using anion substitution and Cl^- channel blockers. However, although K^+ channels accounted for approximately 50 % of the whole cell currents in unstimulated eosinophils, substitution of K^+ with Cs^+ in PMA-stimulated eosinophils had no significant effect on whole cell current activation. Therefore, these results indicated that, in contrast to Cl^- channels, K^+ channel function was not affected by PMA treatment.

Studies of halide permeability sequences can provide important information about the nature of ion channels involved [20]. In this study, the role of different halides in $O_2^{\bullet-}$ production could be described by the sequence $Cl^- \geq Br^- > I^-$. This sequence is different from those reported for CFTR ($Br^- > Cl^- > I^-$) [25] or Ca^{2+}-dependent Cl^- channels ($I^- > Cl^- > Br^-$) [26], but is similar to the permeability sequences of the ClC family ($Cl^- \geq Br^- > I^-$) [27]. The lack of the involvement of CFTR Cl^- channels is further supported by the fact that $O_2^{\bullet-}$ production was insensitive to DPC, an inhibitor of CFTR Cl^- channels [28], and that RT-PCR experiments were unable to detect CFTR mRNA in eosinophils. The lack of CFTR expression in human eosinophils is interesting, since other leukocytes have been shown to express this channel [17]. Similarly, patients with cystic fibrosis appear to lack eosinophils in their nasal polyps, whereas polyps of asthmatics show marked eosinophilia [29].

Presently, nine different ClC genes have been cloned [27]. The results of RT-PCR experiments have shown that only ClC-3 Cl^- channels are expressed in human eosinophils. These channels are thought to be involved in the cell volume regulation [30], but their biophysical characterization remains controversial. While several laboratories have reported the inability to measure ClC-3 currents [27], the results of other studies suggested that ClC-3 channels were: 1) voltage-independent, 2) blocked by DIDS (100 μM), 3) showed outward rectification, 4) had $I^- > Cl^-$ selectivity sequence, and 5) had single channel conductance either ~40 pS [30], or 140 pS [31]. The whole-cell current recorded in our study was voltage-independent, showed some outward rectification, and

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was inhibited by DIDS, suggesting the involvement of ClC-3 Cl^- channels in eosinophil activation. However, the selectivity sequence and estimation of the single channel conductance in our studies were not compatible with the above-published reports. Our studies show $Cl^- \geq Br^- > I^-$ selectivity sequence, which is characteristic for all members of ClC family [27], except ClC-3 [30]. Similarly, channels with the conductance of 40 pS might allow observing single channel events in whole-cell recordings, since eosinophil current was rather small (<50 pA). A 40 pS channel would have single channel current equal to 3.2 pA at the applied voltage of 80 mV. However, current noise recorded at 80 mV was less than 2.0 pA. This suggests that the maximal conductance of the channels contributing to the whole-cell current is 25 pS, and most likely much smaller, since no single channel events could be distinguished in current tracings.

More experiments will be necessary to confirm the involvement of ClC-3 channels in eosinophil activation. However, if the role of ClC-3 channels in eosinophil activation is confirmed, it is likely that ClC-3 channels could also be involved in other processes that are crucial for eosinophil physiology. In particular, migrating eosinophils undergo extensive changes in shape and volume that facilitate extravasation and tissue penetration [8]. It is likely that ClC-3 channels could facilitate this process by regulating cell volume changes.

In conclusion, our findings show that PMA-stimulated $O_2^{\bullet-}$ production is regulated, in part, by the function of Cl^- channels that share some similarities with the members of ClC family. Molecular identification of these channels and understanding of their regulatory mechanisms may help to control the respiratory burst of eosinophils, and, thus serve as a new therapeutic strategy in asthma and related allergic inflammatory conditions.

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ACKNOWLEDGMENTS

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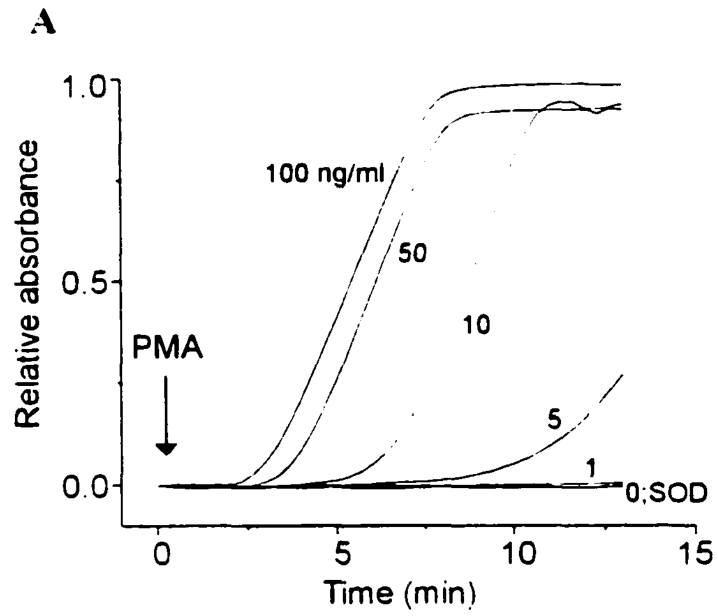
Figure 3.1

A



B

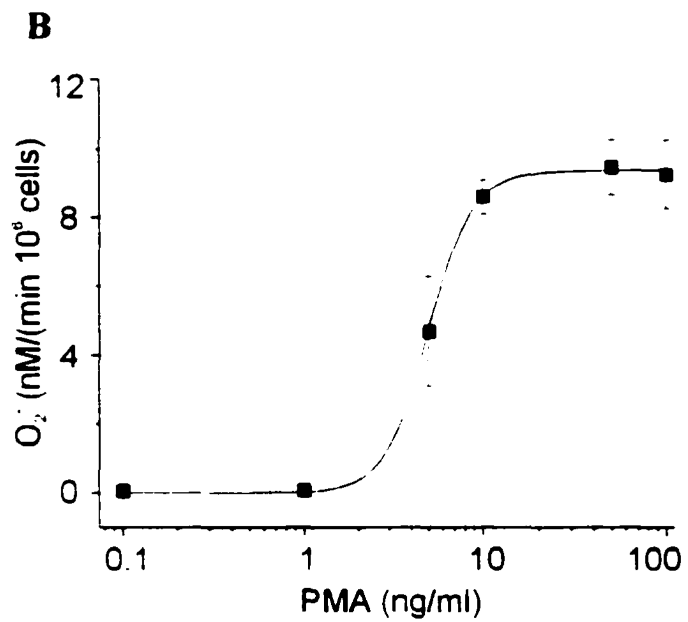


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Figure 3.2

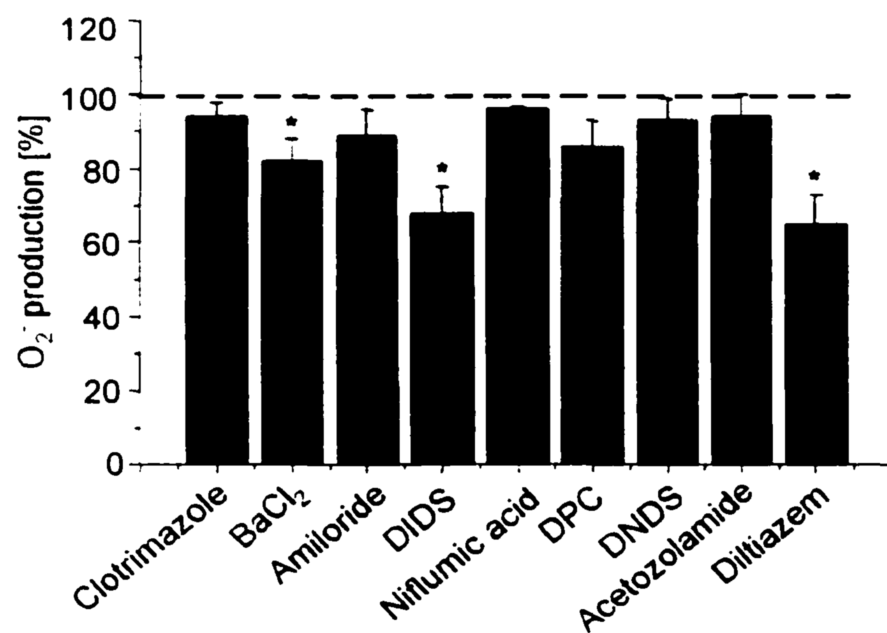

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Figure 3.3

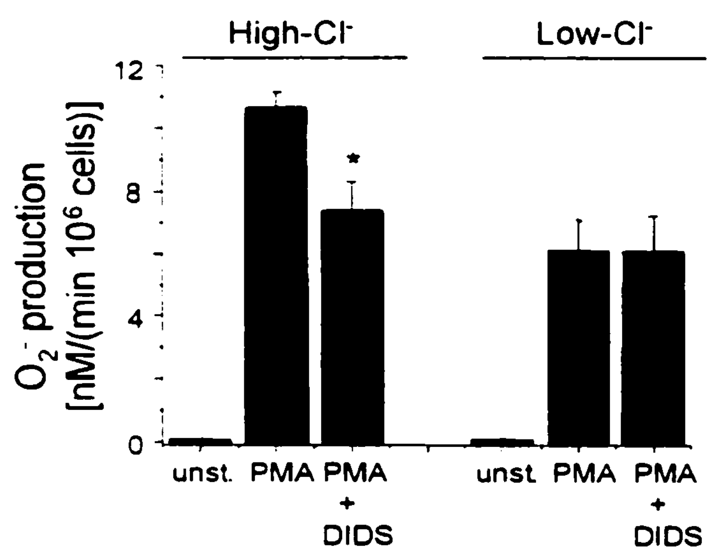

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Figure 3.4

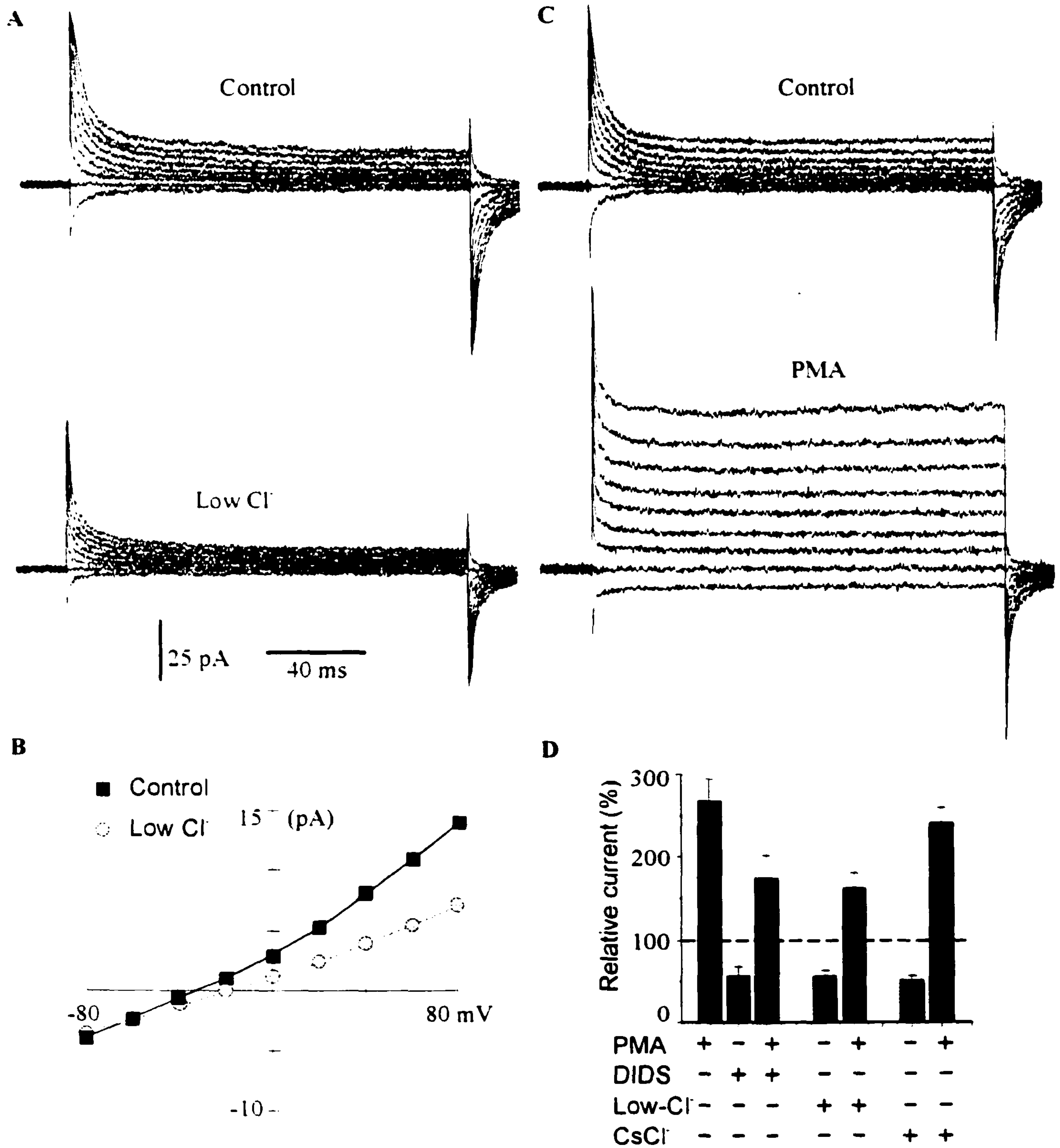

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Figure 3.5

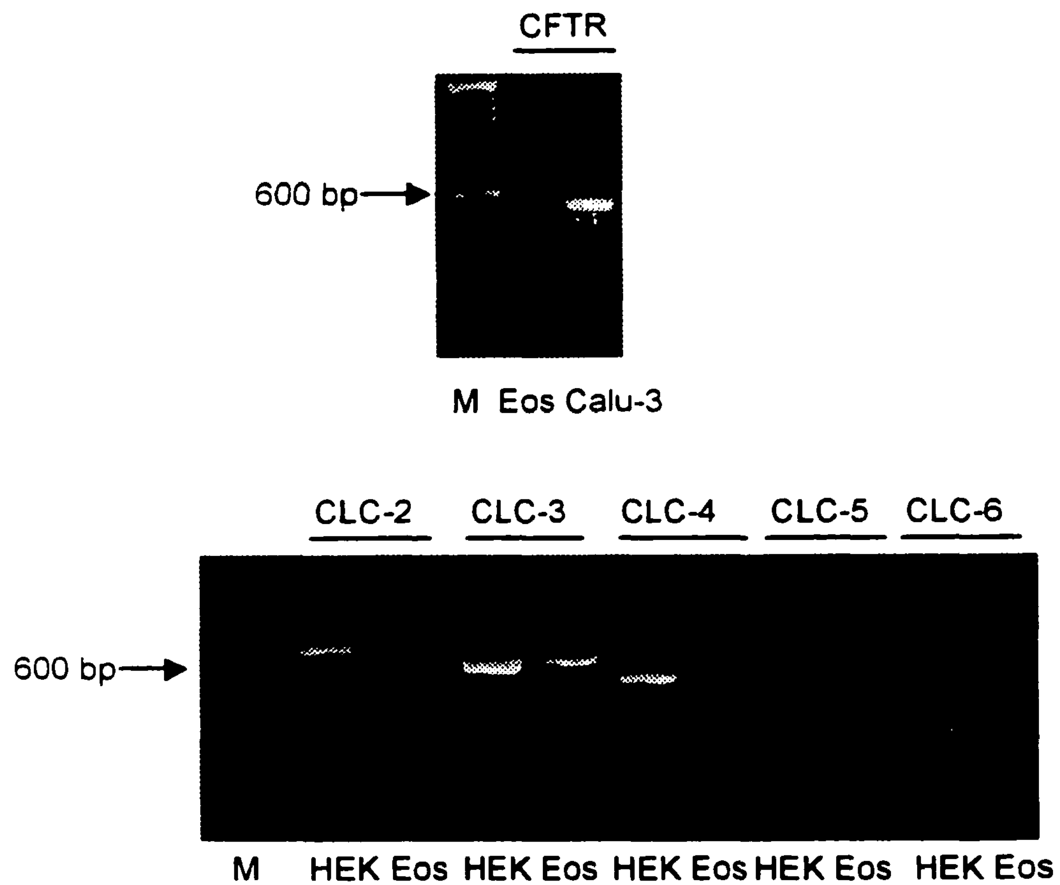

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Table 3.1

The list of the Cl^- channel tested, the positions of the primers relative to the published data sequences, the expected size of the RT-PCR products, and the base sequence of the primers in the 5'→ 3' direction.

Cl^- channel	Primer	PCR product (bp)	Primer sequence
CFTR	1765-1789	555	CATCAGAATCCTCTTCGATG
	2320-2300		GGAATCACACTGAGTGGAGGTCAAC
CLC-2	1079-1102	695	AGCCCTCTTTGTCTACCTGAACCG
	1773-1751		CAGGCAGGTAGGGCAGTTTCTTG
CLC-3	907-930	567	GGGCACTGGCCGGATTAATAGACA
	1473-1450		GTGCACCAAAAGCTACAGAAACCC
CLC-4	684-707	461	GGGGTCTGCCTGTCTGCCTTCTGG
	1144-1121		TCCCGCCTCTTGCCCTCATTCTTG
CLC-5	1362-1385	357	TATAGAGGTACTCGTCGTGACAGC
	1718-1695		AGCTGTTCCATTCCTACTCCTAGA
CLC-6	47-70	344	TCTGTGCTGCTGCTGCAGGTGGTG
	391-368		TGGCTGCACTCCTCCACCGATGTC

The sequences of human Cl^- channels were taken from GenBank under the following accession numbers: CFTR, NM000492; ClC-2, AF026004; ClC-3, X78520; CLC-4, X77197; CLC-5, X91906; CLC-6, NM001286.

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FIGURE LEGENDS

Fig.3. 1

Dose-response curve of PMA-induced $O_2^{\bullet-}$ production in human eosinophils (1×10^6). A: Time course of $O_2^{\bullet-}$ production stimulated by 0-100 ng/mL PMA, added at the time indicated by the arrow. B: Dose-response curve expressed as nM/(min 10^6 cells) and fitted by Eq. 1 with P_{max}= 9.34 nM/(min 10^6 cells), K_d = 4.98 ng/mL, and n = 3.5. Data are plotted as means ± SEM of 3 different experiments.

Fig.3. 2

The effects of channel blockers on $O_2^{\bullet-}$ production. Prior to PMA stimulation (10 ng/mL), cells (1×10^6) were treated with clotrimazole (100 μM, n=7), $BaCl_2$ (5 mM, n=4), amiloride (100 μM, n=7), DIDS (50 μM, n=10), niflumic acid (100 μM, n=3), DPC (1 mM, n=5), DNDS (0.5 mM, n=3), acetozolamide (100 μM, n=5) and diltiazem (50 nM, n=3). Values are expressed as percent of $O_2^{\bullet-}$ production of PMA-treated eosinophils. A significant decrease in $O_2^{\bullet-}$ production was observed in the presence of $BaCl_2$, DIDS and diltiazem ($p<0.05$).

Fig.3. 3

The effect of Cl^- ions on eosinophil $O_2^{\bullet-}$ production. In a high-Cl^- buffer, PMA (10 ng/mL) generated 9.3±0.5 nM/(min 10^6 cells) of $O_2^{\bullet-}$. In the presence of DIDS (50 μM), PMA-stimulated $O_2^{\bullet-}$ production was reduced by 22 % ($p<0.05$, n=10). In a low-Cl^- buffer, PMA generated 6.1±1 nM/(min 10^6 cells) of $O_2^{\bullet-}$, and DIDS had no effect on PMA-stimulated $O_2^{\bullet-}$ production (6.05±1.2 nM/(min 10^6cells), n=3).

Fig.3. 4

A: The effect of Cl^- on the whole cell current in human eosinophils. B: Current-voltage relationships for the recordings shown in panel A. Reducing the Cl^- concentration from 145.1 mM to 11.2 mM, decreased the whole cell current and shifted the reversal potential changed from -32 mV to -19 mV. The holding potential was -60 mV, and 20 mV steps ranging from -80 to +80 mV, were applied every 200 ms. C: Activation of the whole cell

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current by PMA (10 ng/mL). D: The effects of DIDS and ion replacement studies on eosinophil whole cell currents. The mean values of 4-7 recordings obtained from 4 different individuals are shown as percent of whole cell currents in resting eosinophils. PMA increased the whole cell current by 168 % ($p<0.001$). In the presence of DIDS, the whole cell current decreased by 43 % ($p<0.01$), and PMA increased the current by 75 % ($p<0.05$). The replacement of Cl^- with gluconate reduced the baseline current by 43 % ($p<0.01$), and PMA increased the current by 62 % ($p<0.05$). The replacement of K^+ with Cs^+ decreased the baseline current by 49 % ($p<0.01$), but had no effect on the PMA-stimulated current, when compared with the PMA-induced whole cell current in a high-Cl^- solution ($p>0.05$).

Fig.3. 5

A: Lack of CFTR mRNA expression in human eosinophils (Eos). The human airway epithelial cell line Calu-3 was used as a positive control for CFTR expression. B: CLC mRNA expression in human eosinophils (Eos). Eos expressed mRNA for CLC-3, but not for CLC-2, CLC-4, CLC-5 or CLC-6. HEK 293 cells were used as a positive control for CLC expression. The data are representative of 3 different experiments. M represents DNA standard (a 100 bp DNA ladder).

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CHAPTER 4

Nitric oxide activates ATP-dependent K^+ channels in human eosinophils

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INTRODUCTION

Plasma membrane ion channels are involved in stimulus-response coupling in many cell types [1]. However, relatively little is known about their role in eosinophil activation. Ca^{2+}-activated K^+ currents (K_{Ca}) were shown to affect eosinophil superoxide (O_2^-) production [2]. Furthermore, quinidine-sensitive K^+ channels were implicated in promotion of eosinophil shrinkage during apoptosis [3]. Other studies have shown that eosinophils express inwardly rectifying K^+ currents (Kir 2.1) [4], but their functional role is unknown. The involvement of other ion channels, in particular Cl^- channels, in eosinophil activation and O_2^- production has recently been demonstrated [5].

Nitric oxide (NO) is known to regulate ion channels in many cell types. NO activates K_{Ca} and ATP-sensitive K^+ channels (K_{ATP}) in smooth muscle [6], neuronal [7], endothelial [8] and colonic epithelial cells [9] via cGMP-dependent and -independent mechanisms [10;11]. In addition, NO activates CFTR [12] and non-CFTR Cl^- channels in lung epithelial cells [13]. In human airways, several cell types produce NO, including neutrophils [14], macrophages [15], epithelial cells [16], endothelial cells [17], and to a lesser extend eosinophils [18]. An increased amount of NO was found in the exhaled air of asthmatic patients and shown to correlate with lung eosinophilia [19]. NO may also be protective to airways reducing neutrophil recruitment [20] and O_2^- production [21]. However, the intracellular mechanisms that enable NO to exert such a wide range of effects in airways are not completely understood.

The aim of the present study was to investigate the effect of NO on ion channel function in human peripheral blood eosinophils. We hypothesized that endogenous and exogenous NO affects ion channel function via cGMP-dependent and/or -independent mechanisms. We found that endogenous NO did not affect channel function, but NO donors activated whole-cell currents via a cGMP-dependent pathway. Ion replacement studies indicated that the NO-activated current was carried by K^+ ions. Interestingly, NO affected only K_{ATP} channel function, suggesting that these channels may represent a novel target to modulate eosinophil activation in asthma and related allergic diseases.

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MATERIALS AND METHODS

Chemicals and reagents:

Amphotericin B, S-nitroso-glutathione (GSNO), 1-ethyl-2-benzimidazoline (1-EBIO), H89, diazoxide, glibenclamide, clotrimazole and 4-aminopyridine (4-AP) were purchased from Sigma-Aldrich, ON, Canada. S-nitroso-N-acetyl penicillamine (SNAP), N-nitro-L-arginine methyl ester (L-NAME) and 1,2,4-oxadiazole-4,3-quinoxalin-1 (ODQ) were purchased from Alexis CA, USA. 8-Br-cGMP was purchased from Calbiochem, CA, USA.

Patch clamp experiments in human peripheral blood eosinophils:

Peripheral blood eosinophils were purified to homogeneity (<98 %) from atopic asthmatic volunteers who had given their informed consent, as described in Chapter 2 [22;23]. Whole-cell recordings were obtained using the amphotericin B-perforated patch clamp technique. Patch pipettes were pulled from borosilicate glass (A-M Systems, USA) using of a Narishige puller (Tokyo, Japan).

Experiments were performed in the following bath solution: (mM) 137 NaCl, 6.4 KCl, 4.3 Na_2HPO_4, 1.4 KH_2PO_4, 1.2 $MgCl_2$, 0.5 $CaCl_2$, 5 glucose. The pipette tip was dipped into pipette solution: (mM) 137, KCl, 6.4 NaCl, 4.3 $NaHPO_4$, 1.4 KH_2PO_4, 1.2 $MgCl_2$, 0.5 $CaCl_2$, 1 EGTA, 4 glucose. The pipette was then back-filled with the same solution containing amphotericin B (240 μg/mL, Sigma). When the contribution of K^+ channels to the whole-cell current was measured, 137 mM Cs^+ replaced equimolar K^+ in the pipette solution. Similarly, to study the contribution of Cl^- channels, 134 mM gluconate replaced equimolar Cl^- in the bath and pipette solutions. All buffers were supplemented with 0.1 % BSA and the pH was adjusted to 7.4. Experiments were performed at room temperature.

Pipette resistances were between 3-8 MΩ and recordings were obtained using a patch clamp amplifier (EPC-7, List Medical, Germany) in the voltage clamp mode. The

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holding potential was -60 mV, and 20 mV steps, ranging from -80 to +80 mV, were applied every 200 ms. The cells were kept in the bath solution for 1 hour before starting the experiments. After baseline currents stabilized, stimuli or blockers were added to the bath solution and currents were recorded for up to 40 min. Data were analyzed using custom-written patch clamp software (kindly provided by Dr. A.S. French, Dalhousie University). Statistical analysis of whole-cell currents was performed at +80 mV.

RT-PCR:

Total RNA was isolated from $2x10^6$ eosinophils using the Qiagen RNeasy Mini kit (Qiagen). The average amount of RNA obtained from $2x10^6$ eosinophils was 300 ng. One third of the RNA was reverse transcribed using superscript II reverse transcriptase (Gibco) and random hexamers ($50A_{260}$ units, Boehringer Mannheim) as primers. Thereafter, PCR was performed in 20 μL reactions. The expression of K^+ channels was studied using the primer pairs described in Table 4.1. As a positive control for IK, BK and K_v, mRNA from the human airway epithelial cell line A549 was used. As a positive control for TWIK-1 and TASK-2 expression, mRNA from Calu-3 cells was used. One tenth of the cDNA was used in PCR experiments. DNA amplification was obtained by annealing for 45 sec at 64°C for IK and BK, 56°C for K_v, 60° C for TWIK-1 and 51° C for TASK-2. This was followed by an elongation step at 72 °C for 1 min. DNA sequences were amplified during 30 cycles. The sizes of the expected amplified products are shown in Table 1.

Statistical analysis:

Data are presented as means ± SEM; n refers to the number of experiments. In bar diagrams, 100 % represents the amplitude of baseline whole-cell currents at +80 mV. Data are expressed as percentage of baseline currents. The unpaired two-sided Student's

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t-test was used to compare the means of two groups. Values of $p<0.05$ were considered statistically significant.

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RESULTS

NO increases the whole-cell current in human eosinophils

Figure 4.1 shows the effects of two chemically different NO donors, GSNO and SNAP, on the whole-cell current in human eosinophils. In all recordings, the current-voltage relationship (I-V) was obtained in 20 mV steps from -80 mV to +80 mV. Statistical analysis of whole-cell currents was performed at +80 mV. In resting eosinophils, the baseline whole-cell current was 53 ± 13 pA (n=7). GSNO increased the current by 47 ± 7 pA (88%, n=4) whereas SNAP by 59 ± 18 pA (111%, n=4). Similarly, GSNO caused a shift in the reversal potential by -9±2 mV (n=3, $p<0.05$) and SNAP by -12 ± 0.5 mV (n=3, $p<0.05$). This suggests that K^+ channels make a major contribution to the whole-cell current activated by NO.

In order to evaluate the role of endogenous and exogenous NO in the whole-cell current activation, we suppressed endogenous NO production with L-NAME, an inhibitor of nitric oxide synthases. L-NAME had no effect on the baseline current, indicating that endogenous NO did not affect channel function in resting eosinophils (n=4). Subsequent addition of SNAP (100 µM) increased the whole-cell current by 114 ± 10 % (n=4, $p<0.01$), suggesting that exogenous NO mediated this increase.

NO stimulates the whole-cell current via a cGMP-dependent pathway

In order to investigate whether NO was acting on eosinophil ion channels via a cGMP-dependent or –independent mechanism, cells were incubated with 8-Br-cGMP, the soluble guanylyl cyclase (sGC) inhibitor ODQ, or a PKG inhibitor H89, before stimulation with SNAP (Fig. 4.2). ODQ (10 µM) and H89 (1 µM) had no effect on the baseline current and prevented subsequent activation of the current by SNAP (n=5 for both drugs). In other experiments, addition of 8-Br-cGMP significantly increased the whole-cell current, indicating that cGMP is involved in the activation of the observed

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current. The addition of 100 μM and 500 μM 8-Br-cGMP increased whole-cell currents by 27 ± 8 % (n=4), and 124 ± 25 % (n=4). The increase in whole-cell current caused by 500 μM 8-Br-cGMP was similar to that induced by SNAP (139±12 %). All these results suggest that exogenous NO activates the whole-cell current via a cGMP-dependent pathway.

SNAP activates K^+ channels

In order to investigate which ions participate in whole-cell currents evoked by NO, we replaced K^+ or Cl^- ions in the bath solution with Cs^+ or gluconate, respectively (Fig. 4.3). In a gluconate-containing solution, SNAP increased the whole-cell current by 177±25 % (n=4, $p<0.05$), indicating that Cl^- currents do not contribute to the NO-induced current. In contrast, in a Cs^+-containing solution, SNAP was ineffective (n=7, $p>0.05$), indicating that currents activated by SNAP were carried by K^+ ions.

The presence of K^+ channels in eosinophils was investigated using openers of intermediate conductance K_{Ca} channels (IK), 1-EBIO, and K_{ATP} channels, diazoxide (Fig. 4.4). 1-EBIO increased the current by 64 ±20 % (500 μM, n=5) whereas diazoxide by 73 ±19 % (100 μM, n=10), demonstrating the presence of IK and K_{ATP} channels in eosinophils. In the presence of diazoxide, SNAP further activated the whole cell current by 29 ± 10% (100 μM, n=5).

In other experiments, we studied activation of the whole-cell current by SNAP in the presence of different K^+ channel blockers (Fig. 4.4). An inhibitor of K_{ATP} channels, glibenclamide (5 μM and 100 μM), had no effect on the baseline current, but prevented current activation by SNAP (n=6 and n=4, respectively). A blocker of IK channels, clotrimazole, had also no effect on the baseline current (n=6), but it did not prevent current activation by SNAP (n=4, $p<0.05$), indicating that NO had no effect on IK channels. A chemical frequently used to block K_v channels, 4-aminopyridine (4-AP; 2 mM), inhibited the baseline current by 41 ± 5 % (n=7, $p<0.01$), and also prevented its activation by SNAP (n=4).

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Gene expression of K^+ channels in eosinophils

Since K_{ATP} channels have previously been described in human eosinophils [4], we investigated the expression of IK and large conductance (BK) K_{Ca} channels, K_v, and two members of the K_{2P} family, TWIK-1 and TASK-2 (Table 4.1). Figure 4.5 shows representative RT-PCR experiments. Eosinophils express mRNA for IK, K_v and TWIK-1, but not for BK and TASK-2 channels (n=6). The identity of the PCR products was confirmed by sequencing and comparison with the corresponding Genbank sequences.

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DISCUSSION

The central observation of this study is that an increased level of NO activates K_{ATP} channels in human peripheral blood eosinophils via a cGMP-dependent pathway. Eosinophils produce NO in a proinflammatory environment [24], and our aim was to investigate the effects of endogenous and exogenous NO on eosinophil ion channel function. An inhibitor of NO synthases, L-NAME, had no effect on the whole-cell current, indicating that endogenous NO did not affect cell membrane potential and ionic permeability. However, NO donors significantly increased the whole-cell current and shifted the reversal potential towards more negative values, consistent with the activation of K^+ channels.

Activation of soluble guanylyl cyclase (sGC) and generation of cGMP is responsible for many biological effects of NO [13,25]. The results of this study show that the NO/cGMP-dependent pathway is also involved in the regulation of the whole-cell current in eosinophils because, (i) the NO effects could be eliminated by pretreatment of cells with a selective inhibitor of sGC, ODQ, (ii) activation of the whole-cell current by NO was abolished in the presence of a PKG inhibitor, H89, and (iii) application of membrane-permeable 8-Br-cGMP produced an effect similar to that of NO. While these results are consistent with the regulation of eosinophil whole-cell current via the NO/cGMP-dependent pathway, they do not exclude the involvement of a cGMP-independent pathway in this process. The role of a cGMP-independent pathway could be especially important under inflammatory conditions when large amounts of NO are generated, and NO groups could be introduced into some thiol- and transition metals-containing proteins, altering their properties and functions.

The concentration of NO donors used in our study (100 μM) is likely to yield NO concentrations similar to those encountered in native tissues. It is known that alveolar macrophages produce 0.1 nM/(min 10^6 cells) of NO [26], which may generate μM concentrations in the airway surface liquid. Similarly, distal airway fluid of patients with pneumonia contains ~4 μM nitrosothiols [27], and 2-4 μM concentrations of NO were reported in brain during cerebral ischemia [28]. In addition, it has been shown that 100 μM SNAP generates a stable NO concentration of 0.1 μM at 25°C [29]. Therefore, it is

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reasonable to assume that NO amounts used in our study are similar to those found in the native tissue.

The baseline and NO-stimulated whole-cell currents showed a nearly linear current-voltage (I-V) relationship and were neither time- nor voltage-dependent (Fig. 4.1). This suggests that under our experimental conditions a contribution of H^+ channels to the whole-cell current was insignificant, since these channels show time-dependent activation at depolarizing voltages [30]. Our data indicate that Cl^- and K^+ channels dominate the whole-cell current in eosinophils. This conclusion is based on ion substitution studies and the use of ion channel blockers. While Cl^- channels account for ~50 % of the whole-cell current in unstimulated eosinophils [5], substitution of Cl^- with gluconate did not affect current activation by NO, indicating that NO donors activated K^+ but not Cl^- channels.

Human K^+ channel genes can be classified into three groups, based on the similarity in membrane topology [1]. The first group consists of channels characterized by 6 transmembrane domains (6TM), such as K_{Ca} or K_v channels. The second group consists of channels with 4 transmembrane domains (4TM), such as K_{2P} channels. The third group consists of channels with 2 transmembrane domains, such as K_{ATP} channels or G-protein-coupled K^+ channels. Previous studies have shown that eosinophils express mRNA for the K_{ATP} [31] and the voltage-dependent Kir2.1 channels [4]. Therefore, we used RT-PCR to investigate the expression of other K^+ channels. We found that eosinophils express mRNA for the β subunit of K_v channels, Ca^{2+}-dependent K^+ channels (IK), and a member of K_{2P} family, TWIK-1. However, the functional role of K_v channels in eosinophils remains unclear, since the nearly linear I-V relationship suggests that these channels do not make a significant contribution to either the baseline or SNAP-induced current. Similarly, we conclude that the Kir2.1 and TWIK-1 channels do not contribute to baseline or NO-stimulated currents, since both these channels conduct inwardly rectifying currents.

The K_{ATP} channel is a heteromultimeric complex of a K^+-selective pore and a sulfonylurea receptor (SUR) that are structurally unrelated to each other. It is composed of four inwardly rectifying K^+ channel subunits, either Kir6.1 or Kir6.2, and four SUR subunits, which belong to the family of ABC transporters. Different combinations of the

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Kir6.2 or Kir6.1 subunits and the SUR1 and SUR2, account, in part, for the molecular and functional diversity of K_{ATP} channels. Depending on the combination of Kir6.x and SURx subunits, the single channel conductance of K_{ATP} channels has been estimated between 10 and 80 pS in the presence of 140 mM K^+ on both sides of the membrane [32-34].

The results shown in this study suggest that K_{ATP} channels do not contribute to eosinophil baseline currents, since glibenclamide did not affect the cell membrane potential. However, an opener of K_{ATP} channels, diazoxide, and NO donors activated a whole-cell current of ~50 pA, corresponding to a whole-cell conductance of 625 pS at 80 mV. The number of ion channels (N) activated by NO could be evaluated from the following relationship: $G = \gamma N P$, where G – is the whole-cell conductance, γ – is the single channel conductance, and P – is the channel open probability. If we assume that the K_{ATP} channel conductance is 50 pS and that its open probability is $P = 0.5$, then the number of K_{ATP} channels activated by NO is equal to 25.

After activation of K_{ATP} channels by diazoxide, the subsequent addition of SNAP further increased the whole-cell current. Similar effect has been reported for mitochondrial K_{ATP} channels, where NO has been shown to activate K_{ATP} channels and potentiate the effect of diazoxide [35]. It is possible that diazoxide partially opens K_{ATP} channels, and NO is further increasing the channel open probability. Alternatively, eosinophils may possess different subtypes of K_{ATP} channels, and NO might open a different subpopulation than diazoxide.

It is interesting to note that 4-aminopyridine (4-AP), a chemical commonly used to inhibit K_v channels, reduced baseline and NO-induced currents in eosinophils. However, this effect is not likely to be mediated by K_v channels, since K_v current is strongly voltage-gated. A possible explanation for the effects of 4-AP on eosinophil whole-cell current is the relative lack of specificity of this chemical. It has been reported that aminopyridines, including 4-AP, inhibit some but not all voltage-dependent K^+ channels, G-protein-coupled channels, K_{ATP} and BK channels [36-38]. The K^+ channel independent effects of 4-AP include activation of protein kinase C (PKC) [39], allosteric interaction with the muscarinic acetylcholine receptor [40], increase in intracellular pH [38] and Ca^{2+} concentration [41], potentiation of capacitative Ca^{2+} entry [41], and induction of

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apoptosis [42]. Thus, several different mechanisms may underlie the inhibitory effect of this drug on the eosinophil whole-cell current.

The functional role of K_{Ca} channels in eosinophils has been demonstrated in several studies [2;43], but the molecular identity of these channels has not been identified. K_{Ca} channels are classified according to their conductance into large (BK), intermediate (IK) and small conductance (SK) channels. BK channels are abundant in neuron and smooth muscle, IK channels are predominantly expressed in peripheral tissues, whereas SK channels are exclusively expressed in excitable cells [44]. The results of our study show that eosinophils express IK but not BK channels. The presence of functional IK channels was confirmed by showing that 1-EBIO activated the whole-cell current. Interestingly, clotrimazole, a blocker of IK channels, had no effect on the baseline current or its activation by NO, indicating that these channels were likely closed in unstimulated eosinophils and were not affected by NO.

In summary, eosinophils possess several types of K^+ channels but NO affects only K_{ATP} channel function. NO induces an outflow of K^+ ions in eosinophils and thus causes membrane hyperpolarization. This reduces the cytoplasmic Ca^{2+} concentration, and as a result, the eosinophils could be less excitable and stronger stimuli are needed for depolarization. Thus, NO at concentrations likely to be encountered *in vivo* could perform a protective role, preventing eosinophil activation.

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Figure 4.1

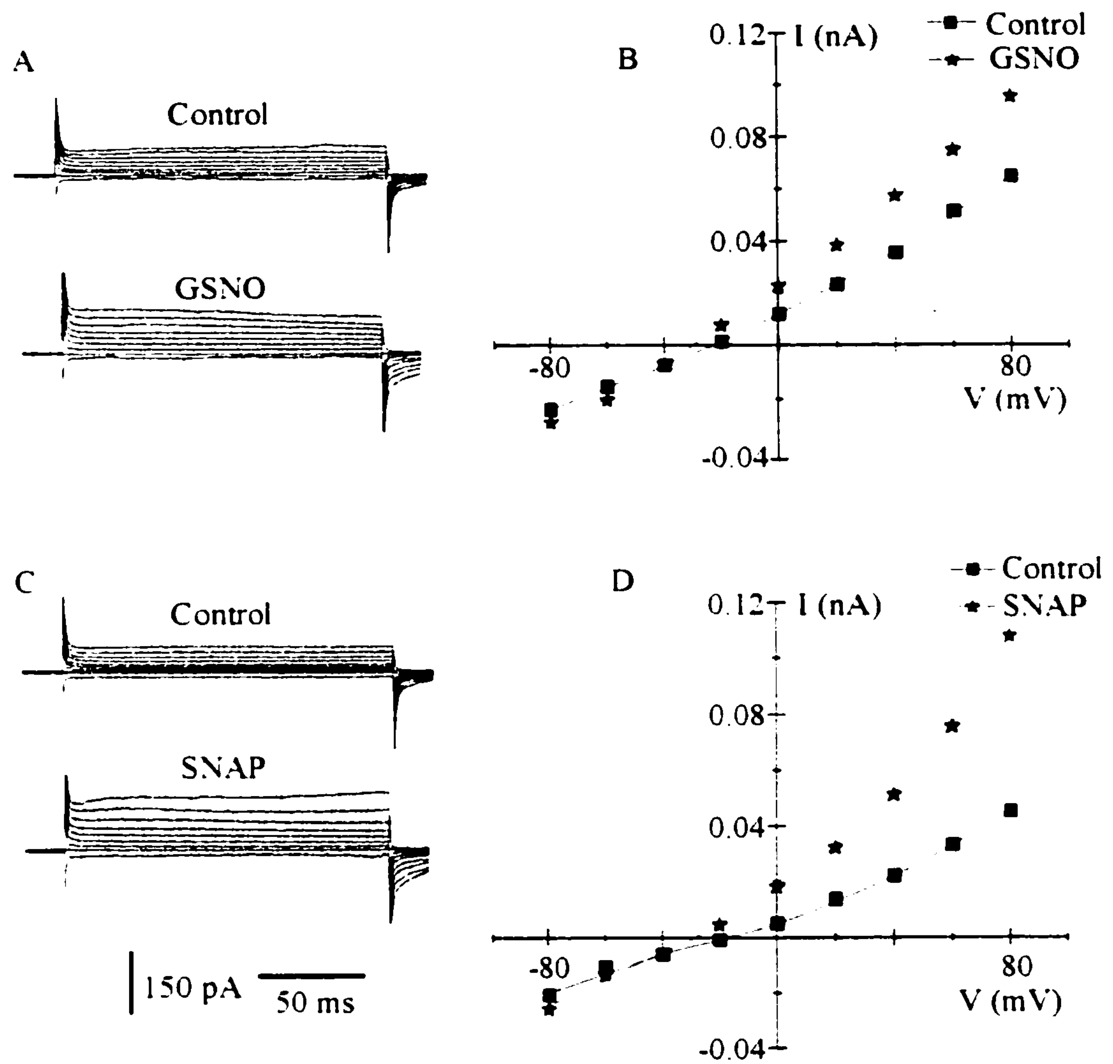

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Figure 4.2

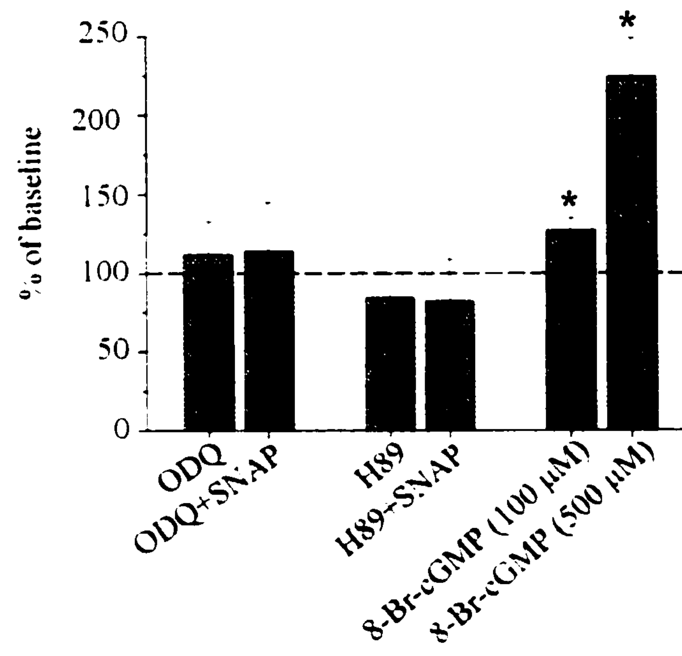

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Figure 4.3

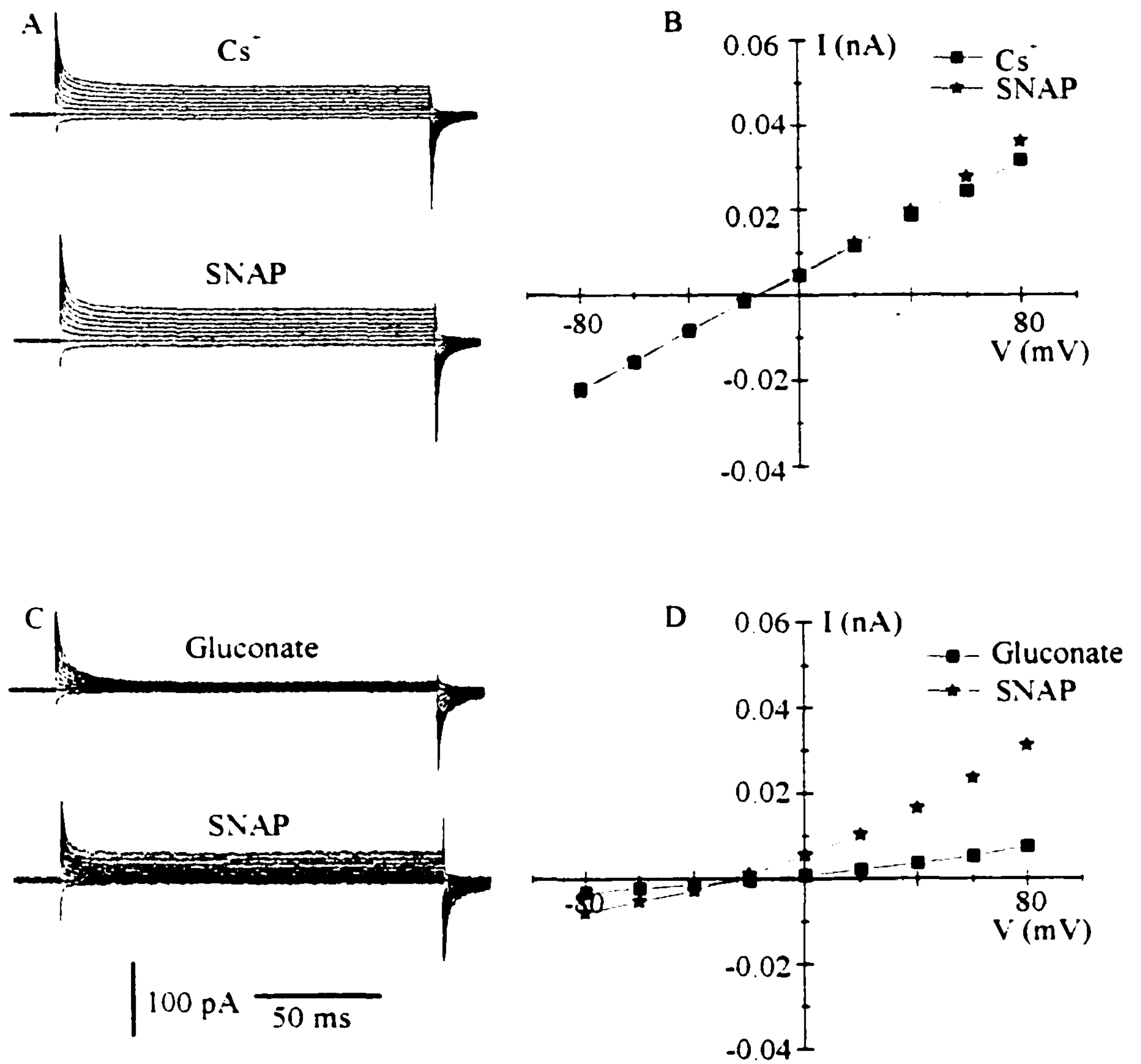

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Figure 4.4

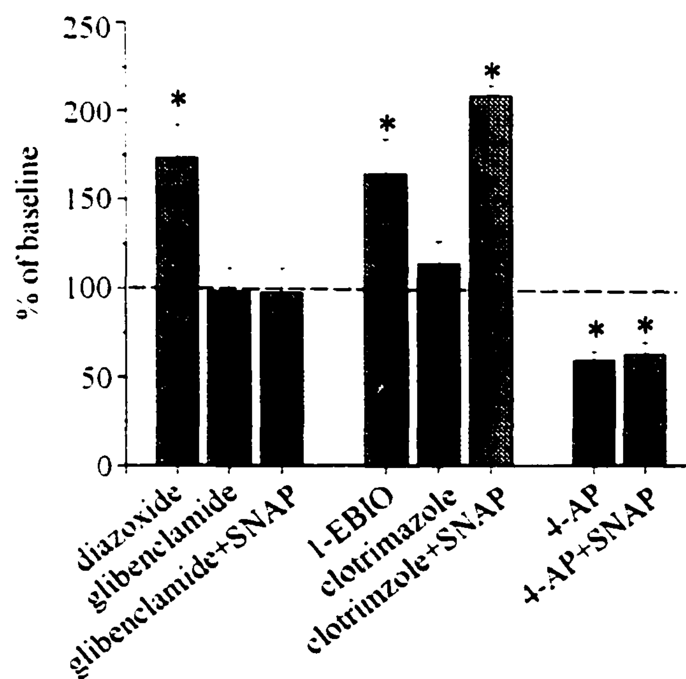

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Figure 4.5

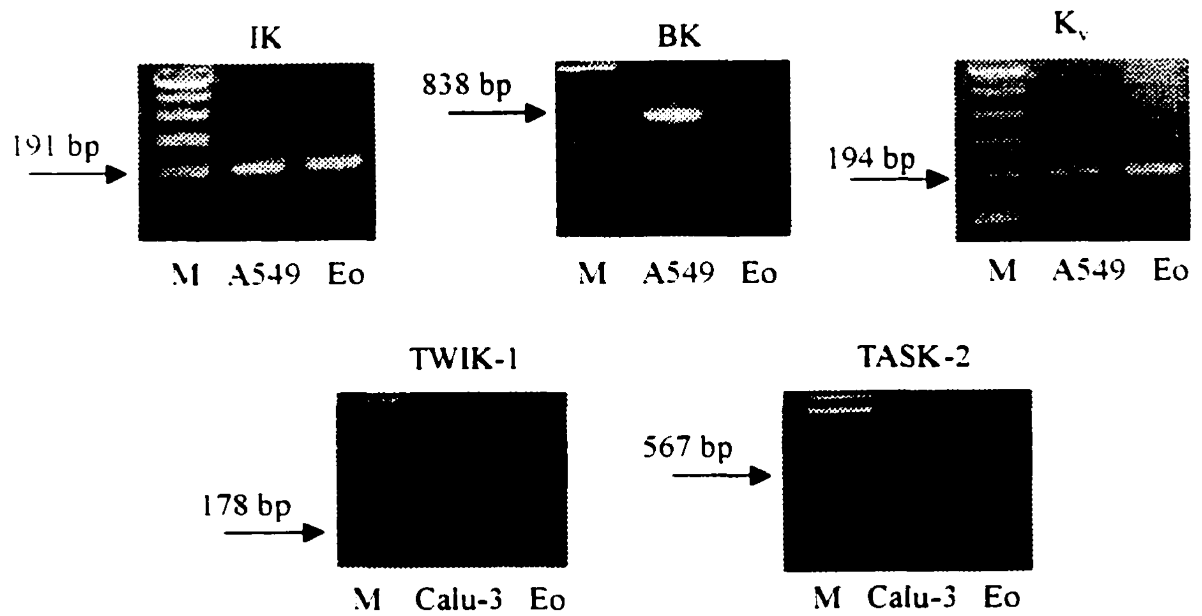

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Table 4.1

List of the K^{+} channels tested, the positions of the primers relative to the published data sequences, the expected size of the RT-PCR products, and the base sequence of the primers in the 5'→ 3' direction.

K^{+} channel	Primer	PCR product (bp)	Primer sequence
IK	703-722	191	GGGCACCTTTCAGACACACT
	893-874		ACGTGCTTCTCTGCCTTGTT
K	68-85	194	ATGCATCTGTATAAACCT
	262-245		TCCATGAACACGTAGAAA
BK	2824-2845	838	CAGCATTTGCCGTCAGTGTCCT
	3683-3662		CATGCCTTTGGGTTATTTTTCC
TWIK-1	1073-1096	178	CCAACTGTCCTTCTCCTCGATCAC
	1250-1230		AGCCTCTTCCTTGCACCCTGA
TASK-2	358-381	567	CTGCTCACCTCGGCCATCATCTTC
	901-924		GTAGAGGCCCTCGATGTAGTTCCA

The sequences of human K^{+} channels are available at the following Genbank accession numbers: IK, AF000972; K_{v}, U16953; BK, U11058; TWIK-1, U33632; TASK-2, AF084830.

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FIGURE LEGENDS

Fig. 4.1

A: Representative recording showing activation of the whole-cell current by GSNO (100 μM). B: Current-voltage relationship of the recording shown in panel A. GSNO shifted the reversal potential from −21 mV to −30 mV. C: Representative recording showing activation of the whole-cell current by SNAP (100 μM). D: Current-voltage relationship of the recording shown in panel C. SNAP shifted the reversal potential from −18 mV to −31 mV.

Fig. 4.2

The NO/cGMP pathway is involved in the whole-cell current activation by SNAP. Baseline currents are expressed as 100 %. ODQ (10 μM) had no effect on the baseline current ($p>0.05$, $n=5$), but inhibited the SNAP-induced increase ($n=5$). Similarly, H89 (1 μM) did not affect the baseline current ($p>0.05$, $n=5$), but inhibited stimulation of the whole-cell current by SNAP ($n=5$). 8-Br-cGMP (100 μM and 500 μM) increased the whole-cell current in a dose-dependent manner by 27 ± 8 % and 124 ± 25 % ($n=4$, $p<0.05$, in each series), respectively

Fig. 4.3

SNAP activates K^+ but not Cl^- channels. A: Representative trace of a whole-cell current using Cs^+-containing pipette and bath solution, showing that the addition of SNAP had no effect on the whole-cell current. B: Current-voltage relationship of the recording shown in panel A.

C: Representative trace of a whole-cell current in the presence of gluconate in the pipette and bath solution, showing that SNAP increased the whole-cell current. D: Current-voltage relationship of the recording shown in panel C.

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Fig. 4.4

Summary of the effects of K^+ channel openers and blockers on the eosinophil whole-cell current. Baseline currents are expressed as 100 %. Diazoxide increased the current by 73±19 % (100 μM, n=10). Glibenclamide (5 μM) had no effect on the whole-cell current but inhibited subsequent current activation by SNAP (n=6 and n=4, respectively). 1-EBIO increased the current by 64±20 % (500 μM, n=5), whereas clotrimazole had no effect on the baseline current (10 μM, n=6) and did not affect subsequent current activation by SNAP (n=4). 4-AP inhibited the baseline current by 41±5 % (2 mM, n=7), and blocked subsequent current activation by SNAP (n=4). * Indicates $p < 0.05$.

Fig. 4.5

Expression of K^+ channels in human eosinophils using RT-PCR. Eosinophils express mRNA for IK, K_v and TWIK-1, but not BK and TASK-2. For IK, BK and K_v A549 cells were used as a positive control. For TWIK-1 and TASK-2 Calu-3 cells were used as a positive control. Data are representative of 6 different experiments. M represents DNA standard (a 100 bp ladder).

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CHAPTER 5

Summary and proposed model for stimulus–secretion coupling in human eosinophils

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The central question in the phenomenon of stimulus-secretion coupling is how does the interaction of a given stimulus with the cell surface translate into membrane potential changes, and ultimately activate or inhibit cellular function? Over the last two decades a vast array of data has accumulated and increasingly refined methods enriched the knowledge about membrane dynamics [1-3]. Undoubtedly, a new impetus was gained when ion channel activities of different cell types became a commonplace and techniques such as patch clamp and flow cytometry became more commonly accessible. Regulated ion channel activity is believed to be the mechanism by which several neurotransmitters, hormones, lymphokines and therapeutic drugs mediate their effects on cell function [4]. Most cell activation processes employ a complicated cascade of second messenger systems, in which Ca^{2+} influx and liberation from intracellular stores often play a significant role [5]. Even in cases where Ca^{2+}-independent pathways are used for signal transduction, changes in membrane potential and ion transport mechanisms are usually involved.

The binding of ligands to their specific surface receptors often affects the molecular structure of the membrane. A signal-induced conformational change of membrane receptors and the activation of ion channels is an almost ubiquitous event in the transduction pathway of extracellular signals that control cell growth, differentiation and activation [6]. The term "cell surface dynamics" refers to a complex pattern of plasma membrane glycoprotein and lipid interactions. The dynamic behavior of the cell membrane includes rotational, translational and segmental movements of molecules, and changes in these parameters frequently have functional implications for the cell [1]. Proteins and other effectors such as hormones and drugs bind to their respective receptors and often trigger local aggregations of particular subunits. They may also induce formation of second messenger molecules via regulation of specific enzyme activities, e.g. the generation of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) from phosphatidyl inositol-4,5-biphophspate (PtdIns-4,5-P_2) by phospholipase C (PLC) [7]. These processes commonly result in an altered transmembrane potential. This may further facilitate conformational changes of membrane proteins and a different accessibility of cell surface molecules to their ligands. The suitable range of membrane

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potential values, allowing the proper intermolecular interactions to occur, is usually limited by a very narrow window. Any significant deviation from this membrane potential window may dramatically decrease the effectivity of transmembrane signaling. For example, sustained and excessive hyper- or depolarization of lymphocytes equally impair signal transduction [8]. In addition to these physical events, several biochemical steps are interwoven into the stimulus-secretion coupling cascade. Among the most important biochemical steps are phosphorylation and dephosphorylation events, which are mediated by kinases and phosphatases, respectively. These reactions can introduce or change the electric charge of molecules, thereby altering their interactions and mobility parameters. The study of stimulus-secretion coupling is further complicated by the fact that in most systems biochemical and biophysical events in a cell change in parallel. Separation of these events into single steps presents itself as an extremely difficult task.

Secretion of vesicular contents is a common feature of excitable (e.g. neurons, pancreatic β-cells) and non-excitable cells (e.g. eosinophils, neutrophils, mast cells) [9-12]. The simplistic view that the universal mechanism controlling secretion is an elevation of $[Ca^{2+}]_i$, whatever the source of this second messenger may be, is no longer tenable in view of reports demonstrating secretion at basal or even negligible $[Ca^{2+}]_i$ levels [13;14]. It is nevertheless commonly accepted that in excitable cells an increase in $[Ca^{2+}]_i$ is a critical triggering event that induces secretion. In non-excitable cells, however, secretion may also be triggered by other second messenger systems, although $[Ca^{2+}]_i$ appears to act as an important regulator in these processes. Given the relative importance of $[Ca^{2+}]_i$ in the regulation of cellular functions in both excitable and non-excitable cells, it is not surprising that several mechanisms are expressed in a cell to regulate $[Ca^{2+}]_i$ levels. In excitable cells, the major pathway for Ca^{2+} entry is activation of voltage-gated Ca^{2+} channels, whereas in non-excitable cells, Ca^{2+} release from intracellular stores appears to prevail (see Chapter 1). Receptor-operated and second messenger-gated Ca^{2+} conductances may also prove to be important in these processes. Many of these mechanisms are regulated by a variety of interactive second messenger systems, which provide the necessary fine-tuning for an appropriate control of $[Ca^{2+}]_i$. However, in non-excitable cells *in vivo*, the question whether an elevation in $[Ca^{2+}]_i$ occurs only as a

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consequence of a ligand-receptor interaction, or is not even necessary for secretion to occur, remains unanswered.

Up to date only few studies have addressed the role of ion channels in stimulus-secretion coupling in eosinophils. Aizawa and coworkers showed a stimulatory role for intracellular Ca^{2+} in human eosinophil EPO release. The effect was amplified by co-administration of the non-hydrolyzable GTP analog GTP-γS, and coincided with an increase in plasma membrane capacitance, indicating granule-fusion events [15]. In addition, to induce maximal O_2^- production physiological concentrations of both intra- and extracellular Ca^{2+} appear to be necessary [16]. PAF- and ionophore-activated K_{Ca} channels have been proposed to play a role in eosinophil MBP release [17]. Hyperpolarization caused by opening of K_{Ca} channels was proposed to increase the electrical driving force for Ca^{2+} entry, via a mechanism similar to that described in the mast cell [18]. It is important, however, to remember that voltage-gated Ca^{2+} channels are closed at hyperpolarizing voltages [19]. Activation of K_{Ca} channels has also been involved in human eosinophil O_2^- generation, although the particular role of K_{Ca} channels in this process has not been clearly defined [20]. An interesting report was published by Stanley and coworkers who proposed a role for intracellular, vesicle-associated K_{Ca} channels in eosinophil exocytosis [21]. They hypothesized that opening of K_{Ca} channels coupled with anion transport across the vesicle membrane would result in an influx of K^+ ions and anions into the vesicle. This would increase the osmotic pressure of the vesicle and cause mediator release. This model suggests a mechanism by which K_{Ca} channels affect degranulation through cell volume regulation. Furthermore, our own data show the presence of K_{ATP} channels in eosinophils and suggest a possible role of these channels in NO-induced responses (Chapter 4).

The few reports available on Cl^- channels in eosinophils include a study describing the inhibitory effect of the Cl^- channel blockers such as nedocromil sodium, NPPB and DIDS on eosinophil cytotoxicity against parasites [22] and on LTB_4-induced O_2^- production [23]. The authors of the latter study also suggested that the dependence of O_2^- production on extracellular Cl^- may be stimulus-specific. From our own results described in Chapter 3 we concluded that activation of Cl^- channels may be an important mechanism for charge compensation during eosinophil O_2^- production.

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In addition to these observations, feedback mechanisms may exist through which eosinophil–derived mediators regulate ion channel function in eosinophils. RANTES and PAF have been shown to open K_{Ca} channels in eosinophils through activation of pertussis toxin-sensitive G proteins via a Ca^{2+}-independent mechanism [24]. Therefore, ion channels might regulate eosinophil mediator release and, in turn, also be regulated themselves by these substances.

In an attempt to gain a better understanding of stimulus-secretion coupling in the eosinophil, and considering the relatively small amount of literature on such events in this cell type, one may try to integrate information obtained from studies in other inflammatory cells. The controversial role of Ca^{2+} and other ions in stimulus-secretion coupling has been well studied particularly in the mast cell. This cell type is thought to be, like the eosinophil, an important secretory cell and one of the key players in allergic inflammation [25]. Mast cells, and the related basophils, initiate allergic reactions by secreting histamine and other inflammatory mediators. The plasma membrane of these cells contains high affinity receptors for the Fc portion of IgE (FcεRI). If a multivalent specific allergen or anti-IgE is added to mast cells primed with the appropriate IgE, the receptors are crosslinked. This leads to fusion of the secretory granule with the plasma membrane and release of granule material [26].

Early experiments showed that Ca^{2+} injection and ionophore-mediated Ca^{2+} influx induce degranulation [27]. This led to the suggestion that control of secretion in mast cells was strictly Ca^{2+}-dependent. Until quite recently, Ca^{2+} channels, like those in neurons, have been postulated to be present in mast cells. Meanwhile, however, it became clear that most likely no voltage-dependent Ca^{2+} channels exist in these cells [28]. It was also shown that changes in the $[Ca^{2+}]_i$ - in comparison to neurons - are much less important for secretion to occur [29]. Simple experiments of loading cells with Ca^{2+}-containing buffers showed a distinct difference between mast cells and neurosecretory cells. Whereas an increase in $[Ca^{2+}]_i$ to approximately 1 μM led to secretion in chromaffine cells, as measured by an increase in cell capacitance, this same level had no effect on mast cells [30], indicating that an increase in $[Ca^{2+}]_i$ is not a sufficient stimulus for secretion to occur in these cells. However, these studies did not contradict earlier reports showing that an increase in $[Ca^{2+}]_i$ could trigger secretion, since in the latter study an

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unphysiologically high concentration of $[Ca^{2+}]_i$ in the range of several μM was used [31]. On the other hand, fixing $[Ca^{2+}]_i$ at basal level using an appropriate Ca^{2+}/EGTA solution did not prevent secretion elicited by compound 48/80 [30], indicating that for this particular stimulus an increase in $[Ca^{2+}]_i$ was not necessary to induce secretion. The authors stressed, however, the fact that these results should not lead to the conclusion that Ca^{2+} is not essential for secretion in mast cells, since when it is completely removed from the medium and not just clamped to basal levels, the secretory response to external stimuli was suppressed. Also, increased $[Ca^{2+}]_i$ dramatically accelerated secretion induced by other stimuli such as GTP-γS, which is known to induce massive and reliable degranulation [32]. It seems likely that GTP-γS and protein kinase C (PKC) activating stimuli such as phorbol esters, render mast cells more sensitive to Ca^{2+}. Nevertheless, it should be kept in mind that without such an additional stimulus, unlike neurosecretory cells, mast cells generally appear relatively unresponsive to Ca^{2+} signals in the physiological range.

Having recognized the importance of basal intracellular Ca^{2+} concentrations in a secretory response, the next question that presents itself is what are the mechanisms that regulate $[Ca^{2+}]_i$? In mast cells, the transient rise in $[Ca^{2+}]_i$ following antigen stimulation appears to be mainly due to IP_3-mediated release from intracellular stores, since a similar signal could be elicited (i) in the absence of extracellular Ca^{2+} and (ii) by direct intracellular application of IP_3 [33]. However, in addition to Ca^{2+} release from intracellular stores, a Ca^{2+} influx mechanism has been proposed. This influx was observed to occur during the falling phase of the IP_3-mediated Ca^{2+} transient. To investigate the nature of this Ca^{2+} influx, voltage steps were applied to the membrane of resting rat peritoneal mast cells, showing that changes in the membrane potential did not affect $[Ca^{2+}]_i$. However, if an external stimulus such as substance P, was applied simultaneously, a release transient was observed which was followed by a secondary increase in $[Ca^{2+}]_i$ that appeared to be voltage-dependent [30]. The increase in $[Ca^{2+}]_i$ was most prominent at negative potentials, since under these conditions the driving force for Ca^{2+} is very high. Two pathways for Ca^{2+} influx were suggested: (i) a non-selective cation channel, which opens and closes randomly and is voltage-independent and (ii) a poorly-defined Ca^{2+}-specific conductance associated with very small currents and activated by external stimuli and IP_3, which

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caused, however, large changes in $[Ca^{2+}]_i$. So far, no single channel currents of this conductance have been recorded. Interestingly, the expression of both conductances varied significantly among cells and animals, and a correlation with the immunological status of the animals was suggested [30]. However, one has to be cautious in concluding from these experiments that the membrane potential plays an important role in stimulus-secretion coupling even in the absence of voltage-gated channels, by affecting the ion flux through non-voltage-operated channels by simply changing the electrochemical driving force for Ca^{2+}. It has to be kept in mind that the intracellular concentration of Ca^{2+} (nM range) is always several magnitudes lower than the extracellular concentration (mM range), and thus a driving force for Ca^{2+} to enter the cell is constantly present throughout the cell activation process [34]. In another study, further evidence for a Ca^{2+}-selective, voltage-independent ion channel was provided [35]. Both, reversal potential and amplitude of the recorded currents were dependent on extracellular Ca^{2+}. Other authors reported the early increase in $[Ca^{2+}]_i$ being IP_3-dependent but independent of extracellular Ca^{2+}, and found that exocytosis was greatly diminished in the absence of external Ca^{2+} [36]. They concluded that extracellular Ca^{2+} is a key modulator of mast cell activation, it greatly enhances cell activation by other stimuli, but this amplification effect as a second messenger differs significantly among cell types and stimuli.

On the other hand, Lindau and coworkers showed that mast cells could evoke large intracellular Ca^{2+} gradients and normally degranulate after antigenic stimulation 1 min after removal of extracellular Ca^{2+} [28]. In the same study, in most but not all cases, antigenic stimulation of mast cells induced a transient Ca^{2+}-activated conductance, which preceded the increase in membrane capacitance representative of degranulation. Interestingly, quinidine and pimozide, inhibitors of Ca^{2+}-activated K^+ channels, abolished the currents without affecting the amplitude and time course of degranulation, indicating that the two observations might represent independent events. Therefore, to date the role of Ca^{2+} in stimulus-secretion coupling is still not completely understood. It seems likely that a minimal concentration of intracellular Ca^{2+} is required for optimal cell activation, but an elevation of $[Ca^{2+}]_i$ – whether caused by Ca^{2+} influx or intracellular release – may under certain conditions neither be sufficient nor necessary for secretion to occur.

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In another non-excitable secretory cell, the neutrophil, the release of granular enzymes, β-glucronidase and lysozyme, as well as the non-granule-associated mediator, O_2^-, was activated by Ca^{2+} release from intracellular stores, but was independent of Ca^{2+} influx [37]. However, other authors reported that for optimal O_2^- production Ca^{2+} originating from an intra- or extracellular source was equally effective, and that decreasing the extracellular Ca^{2+} concentration reduced O_2^- production [38]. Thus, in neutrophils also the role of Ca^{2+} in granule- and non-granule-associated mediator release remains a matter of discussion.

In addition to Ca^{2+} channels, other ionic conductances have been involved in stimulus-secretion coupling. A role for K^+ channels in this process has been proposed by several investigators. At physiological K^+ concentrations of about 130 mM inside and 3 mM outside the cell, the resting membrane potential of cells is strongly negative (around –90 mV). A K^+-selective inward rectifying current with a conductance of 2-3 pS is thought to represent its underlying cause in mast cells [36,39]. Labrecque and coworkers described a different type of K^+ conductance in rat mast cells, which should operate during cell activation [18]. The function of this outward current is thought to damp depolarization during cellular stimulation and therefore increase the driving force for Ca^{2+} influx. Both currents showed differential effects of pertussis toxin, suggesting the involvement of separate GTP-binding proteins in their control. On the other hand, K^+ channel blockers such as quinidine, sparteine and 4-aminopyridine were found to promote histamine release and $^{45}Ca^{2+}$ uptake in peritoneal mast cells [36], suggesting that reduction of K^+ channel activity may facilitate membrane depolarization and thus be a prerequisite for opening Ca^{2+} influx routes. In rat mast cells, depolarization by a high extracellular K^+ concentration was reported to inhibit Ca^{2+} influx and antigen-induced secretion [40]. The authors concluded that changes in the electrochemical gradient for Ca^{2+} are important in determining Ca^{2+} influx and the magnitude of antigen-stimulated secretion from rat mast cells, while the release of Ca^{2+} from intracellular stores is unaffected. Beauvais and coworkers reported that a high extracellular K^+ concentration favored histamine release in one case [41] and inhibited it in the other [42]. The authors suggested that in the former case, high K^+-mediated depolarization decreased the driving force for external Ca^{2+} to enter the cell and consequently decreased IgE-mediated

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secretion. This Ca^{2+} entry was believed to occur through channels opened by an IgE-mediated mechanism. In the second case, a high K^+ containing external solution caused a Ca^{2+}-dependent release of histamine from PMA-stimulated cells, but high extracellular K^+ by itself did not induce release [42]. The authors propose that in this case high K^+-mediated depolarization also decreased the driving force for Ca^{2+}, but may also allow Ca^{2+} to enter the cell by opening voltage-dependent Ca^{2+} channels. However, as described previously, the presence of such channels in non-excitable cells is still controversial. Recently, in human mast cells, a charybdotoxin-sensitive current was described, suggesting the presence of intermediate conductance K_{Ca} channels (IK) [43]. However, charybdotoxin had inconsistent effects on histamine release, indicating that the K_{Ca} channel may enhance, but not be essential, for release of this mediator. In summary, if K^+ is in fact important in stimulus-secretion coupling, its role may most likely be the regulation of membrane potential, which in turn may increase or decrease the electrical driving force for any other ion involved in mediator release.

In addition to mast cells, K^+ currents have also been extensively studied in lymphocytes. Ishida and coworkers found hyperpolarization producing K_{Ca} channels in T cells [44]. These channels showed voltage-dependent opening characteristics, with higher channel activity at lower membrane potentials. Activation of another voltage-gated K^+ channel, $K_v1.3$, has been reported to cause an increase in the extracellular K^+ concentration and subsequently mediate T cell adhesion via β-integrins [45]. Interestingly, $K_v1.3$ and β-integrins could be coimmunoprecipitated, suggesting that their physical association may underlie their functional cooperation. The $K_v1.3$ channel is believed to regulate the resting membrane potential in T cells, and blocking of this channel inhibited a delayed-type hypersensitivity response and thymic development of T cells [46]. In addition, $K_v1.3$ has also been involved in membrane hyperpolarization and in maintenance of an inwardly directed driving force for the secondary influx of Ca^{2+} [47]. Interestingly, this channel has been reported to form a multi-protein complex with calmodulin kinase II, which is known to associate with *src* tyrosine kinases [48]. $K_v1.3$ is a good example for the wide range of effects that activation of one single channel type may exert on cellular function, and prepares us for the complexity which might be

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encountered while dissecting the stimulus-secretion coupling mechanism, a single step at a time.

We have also evidence from mast cell studies for a role of Cl^- channels in stimulus-secretion coupling. Stimulation of rat mucosal mast cells by IgE and antigen or by a monoclonal antibody specific for the high affinity IgE receptor (FcεRI) was reported to result in activation of a Cl^- channel. The conductance of this channel was 32 pS with an increasing open state probability with increasing depolarization [49]. Two compounds, the Cl^- channel blocker 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) and the anti-allergic drug cromolyn, which is a weak inhibitor of mast cell degranulation, showed parallel inhibition of both Cl^- channel activity and mediator release, i.e. serotonin. Interestingly, serotonin release from rat mast cells was found to be strictly dependent on extracellular Ca^{2+} influx [50]. Membrane potential changes of antigen-stimulated rat mast cells showed a biphasic behavior. An initial depolarization attributed to Ca^{2+} influx was followed by a long-lasting repolarization [51]. The authors suggested that this repolarization was caused by FcεRI-mediated activation of Cl^- channels, leading to a decay of the membrane potential to more negative values. A low density of activated Cl^- channels was proposed to be sufficient for this purpose, since their open channel probability is high at the Cl^- equilibrium potential of approximately -45 mV. This mechanism may effectively clamp the membrane potential close to this value in order to maintain Ca^{2+} influx. Inhibition of Cl^- channels may lead to sustained depolarization and thus inhibition of mediator release as shown for serotonin [49]. Neher agrees with the hypothesis that activation of Cl^- channels may be necessary for sustained Ca^{2+} entry [30]. The Cl^- conductance observed in his study was activated by cAMP. However, if applied through the patch pipette, cAMP is known to suppress secretagogue-induced secretion [52]. Therefore, cAMP may have two opposing effects in stimulus-secretion coupling. In the intact cell, the timing and size of a cAMP response may determine which of the two effects predominates. The involvement of Cl^- currents in stimulus-secretion coupling has been confirmed by other studies. Duffy and coworker recently described a slowly activating, Ca^{2+}-independent and outwardly rectifying Cl^- channel, which was stimulated following IgE-dependent mast cell activation and was expressed in about 50 % of human lung mast cells [43]. The authors of this study proposed caution in interpreting the role of

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this Cl^- conductance during cell activation, since the intracellular Cl^- concentration, which varies widely between cells, is not known for human mast cells. The extracellular Cl^- concentration in the tissue has been estimated to be approximately 100 mM [53], and intracellular values of around 30 mM have been proposed for rat peritoneal mast cells [54]. Under such circumstances, Cl^- currents would contribute to membrane repolarization since the reversal potential for Cl^- at these concentrations is about -40 mM, and further indicate a role for Cl^- currents in maintaining Ca^{2+} influx by increasing its electrical driving force. However, if intracellular Cl^- concentrations were similar to those extracellular, then Cl^- channel opening would depolarize the cell and decrease the electrical driving force for Ca^{2+} influx. In such a scenario, with delayed activation of a Cl^- conductance after cell stimulation, it is likely that this Cl^- efflux represents a negative feedback mechanism. The latter may help the cell to recover in between degranulation events [43] Most of these conductances have not been attributed to a specific Cl^- channel type yet. Duffy and coworker suggested that the Cl^- conductance described in their study could be ClC-3, since this channel is widely expressed in mammalian cells and carries an outwardly rectifying current [55]. Interestingly, in human skin mast cells, mRNA expression for ClC-3 and ClC-5 was described [56]

On the other hand, Dietrich and coworker suggested that Cl^- currents might not play a significant role in rat mast cell degranulation [57]. They found that the Cl^- channel blocker 4,4-diisothio-cyanostilbene-2,2'-disulfonic acid (DIDS) blocked a Cl^- outward current in a time- and voltage-dependent manner and, in parallel, inhibited glucosaminidase release upon compound 48/80 stimulation. However, replacement of extracellular Cl^- with glutamate had only a very weak effect on secretion, suggesting that Cl^- currents are not essential for stimulation of exocytosis. This study indicates that care must be exercised in interpreting results obtained using ion channel blockers. Many of these agents may, in addition to ion channels, inhibit other cellular functions independently of ion movement, and thus suggest the involvement of particular ion channels in totally unrelated phenomena.

Similarly to Ca^{2+} channels, Cl^- channels appear to be involved in non-granule-associated mediator release processes such as O_2^- production. Our group proposed a role

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for Cl^- influx, possibly via ClC-3 channels, as a mechanism for charge compensation during depolarization caused by electron efflux via the NADPH oxidase [58].

Reports of a role for H^+ channels in cell activation are mostly limited to O_2^- production, as described in Chapter 1, and such channels are expressed in eosinophils, neutrophils and macrophages [59-61]. Their main function consists in the compensation of loss of negative charge caused by an NADPH oxidase-mediated electron efflux [62]. This would, in addition to Cl^- influx, help to re-establish electroneutrality and counteract the depolarization caused by the electron transport mechanism.

Finally, stimulus-secretion coupling may occur in the absence of activation of any ionic conductance. In fact, Lindau and coworkers showed that the ionic membrane conductance of mast cells remained unchanged after crosslinking IgE receptors under conditions where degranulation occurred in the surrounding cells [39].

In summary, the enigma of the role of ion fluxes in stimulus-secretion coupling remains partly unresolved. Nevertheless, using the information obtained from systems such as the mast cell, lymphocyte and neutrophil, which have hitherto been better studied than the eosinophil, we can start to propose a model of stimulus-secretion coupling in human eosinophils: The first step is usually the interaction of a ligand with its membrane-bound or intracellular receptor (Figure 5.1). Many stimuli including leukotrienes [63], prostaglandins [64], PAF [65], complement factor 5a [66] and chemokines (e.g. RANTES, eotaxin, IL-8) [67] exert their effects via G protein-coupled cell surface receptors (GPCR), as depicted in pathway (1). The large number of GPCRs and their physiological importance is supported by studies performed in GCPR knockout animals [68] and their link to hereditary diseases [69]. Interestingly, GPCR are the target of over 50 % of the currently available therapeutic agents [70]. GPCR are composed of α, β and γ subunits, which upon receptor activation undergo conformational changes leading to the exchange of GDP for GTP at the α subunit. Consequently, the $G\alpha$ and $G\beta\gamma$ subunits stimulate or inhibit effector molecules, including adenylyl cyclase (AC) and PLC [71]. Stimulation of AC increases intracellular cAMP levels causing, among other effects, the activation of protein kinase A (PKA). PLC, on the other hand, cleaves PIP_2 into DAG and IP_3. DAG activates PKC, whereas IP_3 binds to its endoplasmic reticulum receptor and subsequently leads to emptying of intracellular Ca^{2+} stores [72]. Among other effects, an increase in

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$[Ca^{2+}]_i$ activates calmodulin-associated kinase (CaMK) [73]. However, the main result associated with intracellular Ca^{2+} release is cell depolarization. Whether this increase in $[Ca^{2+}]_i$ is supported by influx of extracellular Ca^{2+}, requires further investigation [74;75]. Even in the absence of voltage-gated Ca^{2+} channels on the cell surface of inflammatory cells [76], such an influx may occur via other cell membrane Ca^{2+} channels, e.g. ligand-gated channels or SOC. Membrane depolarization appears to be closely linked to both NADPH oxidase activation and vesicle-associated mediator release. However, the exact relationship between these events is poorly understood and needs further investigation (dotted lines).

Once the membrane is depolarized, and in order for the cell to reassume normal function, the membrane potential needs to return to its resting value, i.e. repolarize. Such a mechanism is usually provided by the activation of K^+ channels on the cell surface [77]. We have shown in Chapter 4 that eosinophils express IK, K_v, K_{ATP} and TWIK-1 K^+ channels, and K^+ extrusion through these channels may help cell repolarization. Particularly K_{Ca} channels could be involved in this process since at the end of cell depolarization the $[Ca^{2+}]_i$ is high. Although the major function of Cl^- channels is to stabilize the resting membrane potential, a small contribution to the membrane repolarization could derive from activation of these channels. If in fact Cl^- channels participate to a certain degree in cell repolarization, this effect is most likely exerted by Ca^{2+}-activated Cl^- channels (CaCC), since at the end of the depolarization process the $[Ca^{2+}]_i$ is high. This would favor the opening of these channels [78]. In contrast, ClC channels are less likely to be involved in membrane repolarization. Although these channels are activated by high $[Ca^{2+}]_i$ [79], as found during cell activation, eosinophils express only ClC-3 (as described in Chapter 3), a channel which is closed at depolarizing membrane potentials [55]. The depolarized membrane potential provides the necessary driving force for K^+ to leave and Cl^- to enter the cell. In summary, K^+ efflux, whether or not supported by a certain amount of Cl^- influx, will result in an increase in intracellular negative charges and therefore membrane repolarization.

Protein kinases have been described in numerous systems to activate K^+ and Cl^- channels [80;81]. Regulation of K^+ channels may occur via protein kinase-dependent pathways, including PKA, PKC, PKG, CaM-dependent kinase and MAPK kinases.

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Binding to tyrosine kinase-associated receptors and activation of the MAP kinase-signaling pathway, as shown in Figure 5.1, pathway (2), is employed by stimuli such as immunoglobulins [82], most interleukins [83] and growth factors [84]. Furthermore, Ca^{2+} channel activation may also be supported by such phosphorylation processes since, for example, ryanodine receptor activation in T cells has been reported to be tyrosine phosphorylation-dependent [85]. Other possible ion channel activators are membrane permeable compounds such as NO, which diffuses across the membrane barrier and activates ion channel proteins either directly via nitration or nitrosylation processes or indirectly via cGMP-mediated stimulation of PKG [86;87]. This is depicted in Figure 5.1, pathway (3). As a matter of fact, in eosinophils, NO activates K_{ATP} but not Cl^- channels, as described in Chapter 4. Finally, once vesicle- and non-vesicle-associated mediators are released, these substances may by themselves regulate ion channel function.

To conclude, most investigators agree that an elevation of $[Ca^{2+}]_i$ represents an important step in inflammatory cell mediator release. Intracellular Ca^{2+} is, in many systems, necessary for fusion of mediator-containing vesicles with the plasma membrane and optimal generation of non-vesicle-associated mediators such as O_2^-. In the case of NADPH oxidase activation, H^+ efflux results in charge compensation [60], but Cl^- channels may play a supportive role in eosinophils [58]. Thus, stimulus-secretion coupling in inflammatory cells resembles a complex network of interactions between intracellular second messenger systems and ion channel activation and inactivation. Different pathways employed by different stimuli may ultimately lead to a common outcome, cell activation and mediator release.

After reviewing the literature it appears increasingly evident that the role of ion channels in inflammatory cell activation and mediator release is far from being completely understood. Generally, future studies will be necessary to identify at a molecular level the ion channels expressed in inflammatory cells, and how they correlate with stimulus-secretion coupling processes. In particular, the presence or absence and the functional role of voltage-gated Ca^{2+} channels in non-excitable cells, including eosinophils and neutrophils, has to be confirmed. In addition, it is important to screen for other Ca^{2+}-permeable conductances such as SOC or non-selective cation channels on the

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plasma membrane of these cells and to clarify whether plasma membrane Ca^{2+} channels can "crosstalk" with Ca^{2+} channels in the endoplasmic reticulum.

Furthermore, considering the large variety and importance of K^+ channels identified in other cell systems, it is unlikely that we have a complete knowledge of all K^+ channel subtypes functioning in inflammatory cells. Once other K^+ channels are described at molecular level in these cells, another difficult but nevertheless important task would be to elucidate the specific functions of these channel subtypes. A small, but important step towards a better understanding of K^+ channel activation in eosinophils is outlined in Chapter 4, where I described the presence of IK, K_v and TWIK-1 K^+ channels in this cell type as well as the activation of K_{ATP} channels by NO.

Literature on Cl^- channels in inflammatory cells is particularly scarce. Further studies will be necessary to clarify the role of Cl^-, the most abundant anion in nature, in inflammatory cell activation. My own work focused on identifying the Cl^- channels expressed in human eosinophils where a role for these channels in O_2^- production was proposed. However, different Cl^- channels may be expressed in other inflammatory cells and serve different purposes. Furthermore, the role of Cl^- channels in eosinophil mediator release other than O_2^- needs to be addressed.

In summary, I believe that we stand at an important juncture in this fascinating field where future studies leading to a better understanding of ion channel function in stimulus-secretion coupling in eosinophils appear crucial in the search for new therapeutic strategies against eosinophil-associated diseases.

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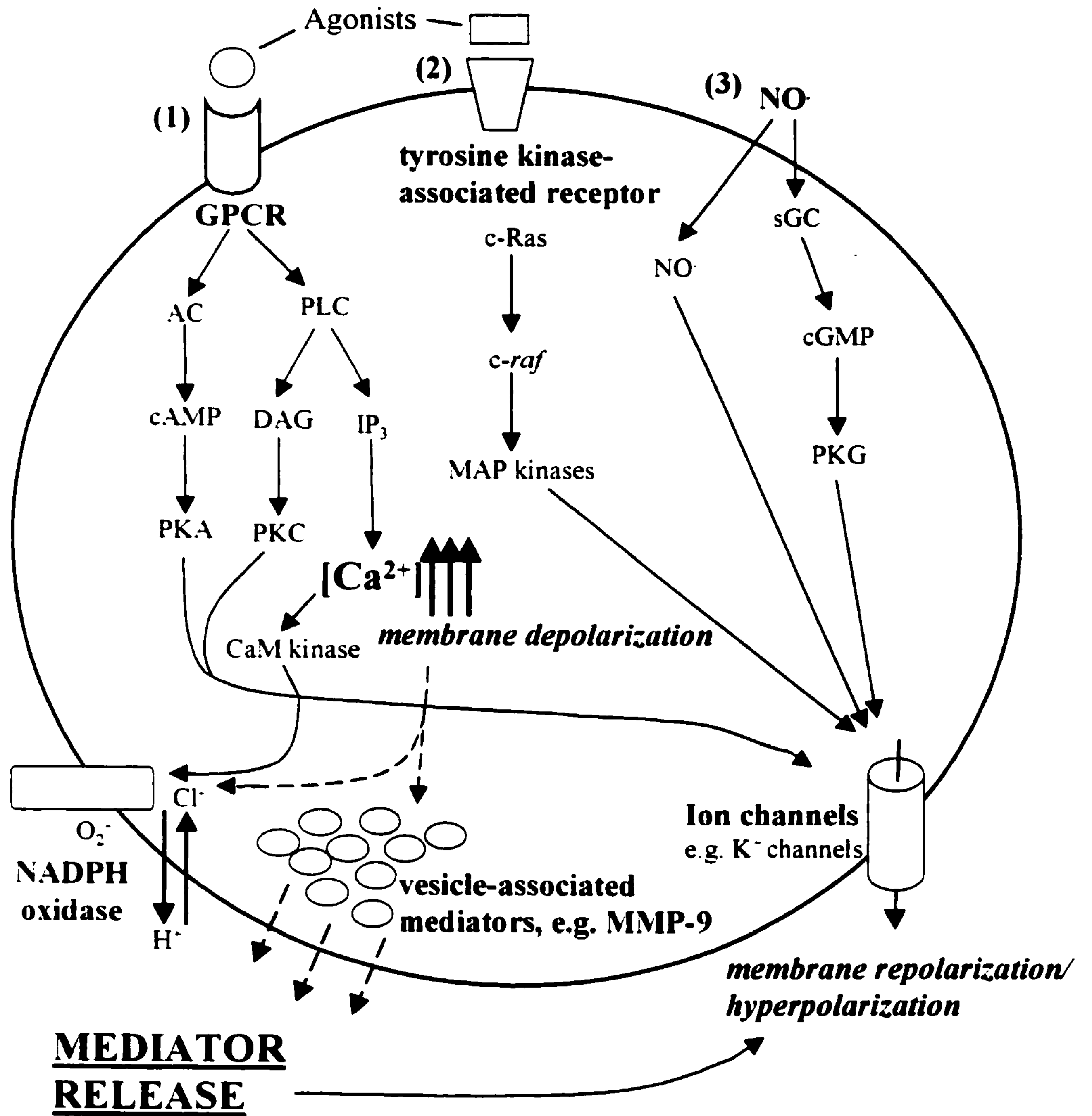
Fig.5.1



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LEGENDS TO FIGURES

Fig.5.1

A schematic illustration of possible mechanisms associated with stimulus secretion coupling in eosinophils. Agonist-induced stimulation of plasma membrane receptors or direct diffusion of reactive oxygen species such as NO into the cell can activate numerous intracellular signaling pathways. Stimulation of GPCRs can lead to activation of PKA, PKC and IP_3, whereas stimulation of tyrosine kinase associated receptors commonly activates the MAP kinase pathway. Cell activation leads to an increase in $[Ca^{2+}]_i$ and to membrane depolarization. These events are commonly associated with cell degranulation and activation of the NADPH oxidase, but the underlying mechanisms are poorly understood (dotted lines). Subsequent activation of K^+ channels contributes to membrane potential repolarization. Activated protein kinases may modify ion channel function via phosphorylation processes. Reactive oxygen metabolites can interfere with channel function either directly or via activation of a cGMP/PKG-dependent pathway. Also eosinophil-derived mediators may regulate plasma membrane ion channels.

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