REGULATION OF INTRACELLULAR FREE CALCIUM AND ELECTROPHYSIOLOGICAL PROPERTIES OF CULTURED HUMAN MICROGLIA

by

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Abstract

This study was performed to investigate the changes in the concentration of free calcium ($[Ca^{2+}]_i$) in cultured human microglia in response to ATP and cholinergic agonists by using calcium microfluorescence imaging measurements with the fluorescent dye fura-2. Whole-cell patch-clamp recording was also used to study the effects of ATP on the properties of membrane currents of human microglia. Microglia are the representatives of macrophages in the brain, and serve specific functions in the defense of the central nerves system (CNS) against microorganisms. An important function of microglia in the CNS is a rapid response to neuronal damage, thus, they can be mobilized rapidly to deal with different pathological conditions. The application of ATP (100 μM) to microglia led to a transient $[Ca^{2+}]_i$ increase with two components, an initial increase and a delayed plateau. The initial increase was also observed in Ca^{2+}-free media suggesting it is due to the release of Ca^{2+} from internal stores. The elevated $[Ca^{2+}]_i$ plateau is due to Ca^{2+} influx. The application of either 100 μM carbachol (CCh), or 100 μM acetylcholine (ACh) to microglia elicited a transient $[Ca^{2+}]_i$ increase which was also composed of two phases, an initial spike and a sustained plateau. This initial spike also appeared in the Ca^{2+}-free recording buffer. The CCh-induced $[Ca^{2+}]_i$ increase was atropine-sensitive, and was largely mediated by release of Ca^{2+} from internal stores, followed by Ca^{2+} influx. Both the ATP- and CCh-produced $[Ca^{2+}]_i$ increases in microglia desensitized to repeated agonist exposures. ATP (100 μM) produced a transient activation of an inward non-selective cation current at a holding potential of –60 mV. The ATP responses in whole-cell recording also included an enhanced transient expression of an outward K^+ current activated with applied depolarizing steps and an increase in inward K^+ conductance. When ATP or CCh exposures were performed in the absence of external Ca^{2+}, adding back external Ca^{2+} always produced a large

$[Ca^{2+}]_i$ increase. Similar observations were made when the store depleting membrane ATP-ase inhibitor thapsigargin (Tg) was applied to the cell in Ca^{2+}-free conditions or by incubating the cell in Ca^{2+}-free buffer for a few minutes. This external Ca^{2+} addition induced a $[Ca^{2+}]_i$ increase suggesting that capacitative Ca^{2+} entry mechanisms exist in human microglia. Our results demonstrate $[Ca^{2+}]_i$ responds to extracellular ATP and cholinergic agonists. We also find expression of depolarizing inward cation currents and outward K^+ currents to ATP which are not present in control condition. These data suggest that ATP and cholinergic agonists could act as signals to stimulate the transition of microglia from a resting state to an activated state under some circumstances in the CNS, and could be involved in the pathology of CNS disease.

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List of Abbreviation

$[Ca^{2+}]_i$	Intracellular free calcium concentration
$[Ca^{2+}]_o$	Extracellular free calcium concentration
Aβ	β-amyloid
ACh	Acetylcholine
AD	Alzheimer's disease
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CaR	Calcium-sensing receptor
CCh	Carbachol
CICR	Calcium-induced calcium release
CIF	Calcium influx factor
CNS	Central nervous system
CPA	Cyclopiazonic acid
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
EGTA	Ethylene glycol-bis(β-aminoethyl ether) tetraacetic acid
ER	Endoplasmic reticulum
Fura-2/AM	Fura-2 acetoxymethyl ester
GM-CSF	Granulocyte-macrophage-colony-stimulating factor
HBSS	HEPES buffered Hank's balanced salt solution
I_{CRAC}	Calcium-release-activated or depletion-activated calcium channel
IL-1	Interleukine 1
INF-γ	Interferon gamma
$InsP_3$	Inositol 1,4,5-triphosphate
$InsP_3R$	Inositol 1,4,5-triphosphate receptor

K_d	Dissociation constant
LPS	Lipopolysaccharide
mAChR	Muscarinic acetylcholine receptor
M-CSF	Macrophage colony-stimulating factor
mRNA	Messenger ribonucleic acid
NO	Nitric oxide
P_2	Purinergic receptor type 2
PAF	Platelet-activating factor
PBS	Phosphate-buffered saline
PKC	Protein kinase C
PLC	Phospholipase C
PMCA	Plasma membrane Ca^{2+}-ATPase
R	Ratio
R_{max}	Maximum ratio
R_{min}	Minimum ratio
ROC	Receptor-operated channel
RYR	Ryanodine receptor
SEM	Standard error of the mean
SERCA	Sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase
SOC	Store-operated calcium channel
SP	Senile plaque
SR	Sarcoplasmic reticulum
Tg	Thapsigargin
TGF-β	Transforming growth factor
TNF-α	Tumor necrosis factor alpha
VDCC	Voltage-dependent calcium ion channel

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Chapter One INTRODUCTION

1. Properties of microglia

1.1 Origin of microglia

It has become apparent in recent years that microglia are the representatives of macrophages in the brain. Macrophages are differentiated cells which, in turn, are derived from monocytes. Monocytes form in bone marrow and remain in circulation for a short term before entering the connective tissue, where they differentiate into macrophages and other mature components of the mononuclear phagocyte system. Microglia were first described in 1919 by Del Rio Hortega. Through studies of the morphology of microglia and their entry into brain in late embryonic life, he demonstrated the mesodermal origin of microglia, and suggested that they become activated, or phagocytotic, in response to injury or inflammation in the brain (Del Rio-Hortega, 1919). Later, other researchers suggested that microglia were of neuroectodermal origin, lacked phagocytotic capability (Fujita and Kitamura, 1975; Dolman et al., 1991), and were related to glia in lineage. Recent studies have done much to confirm Hortega's view of the mesodermal origin of microglia from the monocyte-macrophage cells which enter the brain during embryonic and early postnatal life. For example, it has been shown that microglia express typical leukocyte surface proteins such as leukocyte common antigen (Itagaki et al., 1988), MHC class I and class II glycoproteins (Tooyama et al.,1990; McGeer et al., 1987), β-2 intergrins (Akiyama and McGeer, 1990), and immunoglobin receptor $Fc\gamma R_1$, which is specific for monocytic cells (Akiyama and McGeer, 1990).

Since the early 1980s, microglia were widely accepted as the immunocompetant cells of the central nervous system (CNS). As with circulating monocytes, activated microglia show

upregulation of many plasma membrane surface antigens like MHC class I antigens. In addition, microglia have phagocytic potential, such as respiratory burst capability which permits these cells to generate oxygen free radicals, a feature often seen in monocytic cells. Cultured microglia have been shown to be capable of producing superoxide anions (Giulian and Baker, 1986). A comparison between the NADPH-dependent respiratory burst activity in cultured brain microglia and peritoneal macrophages following a variety of stimulants show that these two related cell types possess comparable activity (Klegeris and McGeer, 1994). Thus, evidence from several sources supports the conclusion that brain microglia are representatives of the monocytic system, and that they are ontogenetically related to cells of the mononuclear phagocyte lineage, unlike all other cell types in the CNS.

1.2 Microglia function in the brain

Microglia work as brain macrophages serving specific functions in the defense of the CNS against microorganisms. As professional phagocytes, microglia can destroy invading micro-organisms, and remove debris from brain tissue in neurological disease and during normal development (Kreutzberg, 1996). In purified cultures of microglia, two different morphological forms can be distinguished, a ramified form having extensively branching processes, and an ameboid form, which is round with a flat halo around the soma (Frei et al., 1987; Giulian and Baker, 1986). The ramified microglia were thought to be in a resting state, and the ameboid microglia in an activated functional state. Any change in the microenvironment of the brain, such as infection, inflammation, trauma, ischemia and neurodegeneration can activate microglia (Streit et al., 1990). Activated microglia respond to various stimuli and are involved in autoimmune inflammatory disorders of the brain. In general, it would be expected

that an important property of microglial function in the CNS would be a rapid response to neuronal damage. Thus, microglia may also be considered to serve protective and supportive functions in the brain and can be mobilized rapidly to deal with different pathological conditions.

At present, little is known about how microglia respond to various stimuli, however, the response is likely diverse, and may involve several components. In fact, microglia are extraordinarily sensitive to changes in the brain microenvironment, whatever the nature of the exciting mechanism or substance. Microglia respond not only to changes in the brain's integrity but also to more subtle alterations, such as an imbalance in ion homeostasis that precedes pathological changes. For example, in cultured microglia, several soluble inflammatory mediators such as cytokines and bacterial products like lipopolysaccharide (LPS) have been demonstrated to induce a wide range of microglial activities. These activities include increasing the amount of phagocytosis, chemotaxis, activation of the respiratory burst and release of cytotoxic substances *in vitro.* Cytotoxic substances released by microglia include free oxygen intermediates, nitric oxide (NO) and cytokines (Colton and Gilbert, 1987) like interleukin-1 (IL-1) (Giulian et al., 1996), macrophage colony-stimulating factor (M-CSF) (Ravich et al., 1991) and tumor necrosis factor (TNF) (Merrill, 1992). Microglia can also produce amyloid precursor protein (APP) (Banati et al., 1995). Purified human microglia significantly increase the synthesis of quinolinic acid, an excitatory amino compound, following activation by either LPS or interferon-γ (INF-γ) (Espey et al., 1997). As microglial activation occurs in some CNS disorders, understanding the molecular mechanisms of microglial activation may lead to new treatment strategies for CNS diseases.

Interestingly, the cytotoxic properties of microglia can be modulated by neurotransmitters and cytokines (Loughlin et al., 1993). Studies have shown that the function of

microglia is related to their transformation from the resting state to an activated state. Microglia appear to detect signals from neurons, like adenosine triphosphate (ATP), high K^+, abnormal amounts of acetylcholine (ACh), or the release of cytokines. By either electrophysiological studies or fura-2 fluorescence imaging, cultured mouse microglia have been shown to possess receptors for CNS signaling molecules like ATP (Walz et al., 1993; Langosch et al., 1994), ACh and noradrenaline (Whittemore et al., 1993). Noradrenaline reduces the production of IL-1 and free-radicals in macrophages, and β-adrenergic agonists like isoproterenol reduce production of IL-1 and TNF-α in microglia. The ability of microglia to respond to molecules involved in neurotransmission allows them to monitor the physiological integrity of their surroundings and to react quickly in the event of a pathological disturbance. In effect, neuronal activity could control microglial activation. While cytokines, such as interferon-γ (INF-γ) prime microglia to be activated, other cytokines such as transforming growth factor-β1 (TGF-β1) or IL-4 down-regulate microglial cytotoxicity. Microglia also secrete β-amyloid and INF-γ which both exert synergistic effects *in vitro*, most likely acting in rodents through the release of TNF-α and reactive nitrogen intermediates (Meda et al., 1995)

Because of the difficulty in measuring microglial function *in vivo*, we still do not fully understand the function of these cells in the normal brain. However, it is likely that rapid activation of microglia in pathological events might effect the CNS *in vivo*.

1.3 Microglia in neurodegenerative disease

A number of researchers have identified activated microglia in the nervous system of patients having neurological diseases, including Alzheimer's disease (AD) (McGeer et al.,

1987), amyotrophic lateral sclerosis (Kawamata et al., 1992), multiple sclerosis (Boyle and McGeer, 1990), Parkinson's disease (McGeer et al., 1988), and acquired immuno-deficiency syndrome (AIDS)-related encephalopathy. Since non-activated microglia are always found in the area of amorphous plaques or benign lesions (McGeer et al., 1994), it has been suspected for many years that microglia are associated with the amyloid plaque pathology in AD. This suggested that the presence of microglia may be disease-specific rather than related to the stage of microglial function. Only recently have studies clearly demonstrated the presence of activated microglia in neurodegenerative diseases (Eikelenboom et al., 1994).

One key component of the inflammatory responses in AD is the presence of activated microglia associated with the senile plaque (SP) lesions (Mattiace et al., 1990). One experiment has suggested that cerebrospinal fluid from AD patients contains antibodies against amoebiod microglia, specifically, those microglia expressing MHC class II antigens (McRea et al., 1997). The neurolopathological features of AD include complex aggregations of proteins composed primary of a distinctive peptide, β-amyloid (Aβ). β-amyloid is an abnormal cleavage product of the large amyloid precursor protein (β-APP), a highly conserved secretary glycoprotein expressed at high levels in mammalian brain by neurons, astrocytes, and activated microglia. Aβ is a peptide 42 amino acids in length, and its deposition in SP of AD is thought to be secondary to overproduction of β-APP. It is generally believed that Aβ is in some way responsible for the synaptic and neuronal loss in AD (Younkin, 1995). Exposure of microglia to Aβ peptides can induce the release of cytokines and complement components. There are also reports showing that murine microglia internalize micro-aggregates, or Aβ peptide (Paresce et al., 1996). This uptake is reduced by co-incubation with scavenger receptor ligands, and suggests that microglia

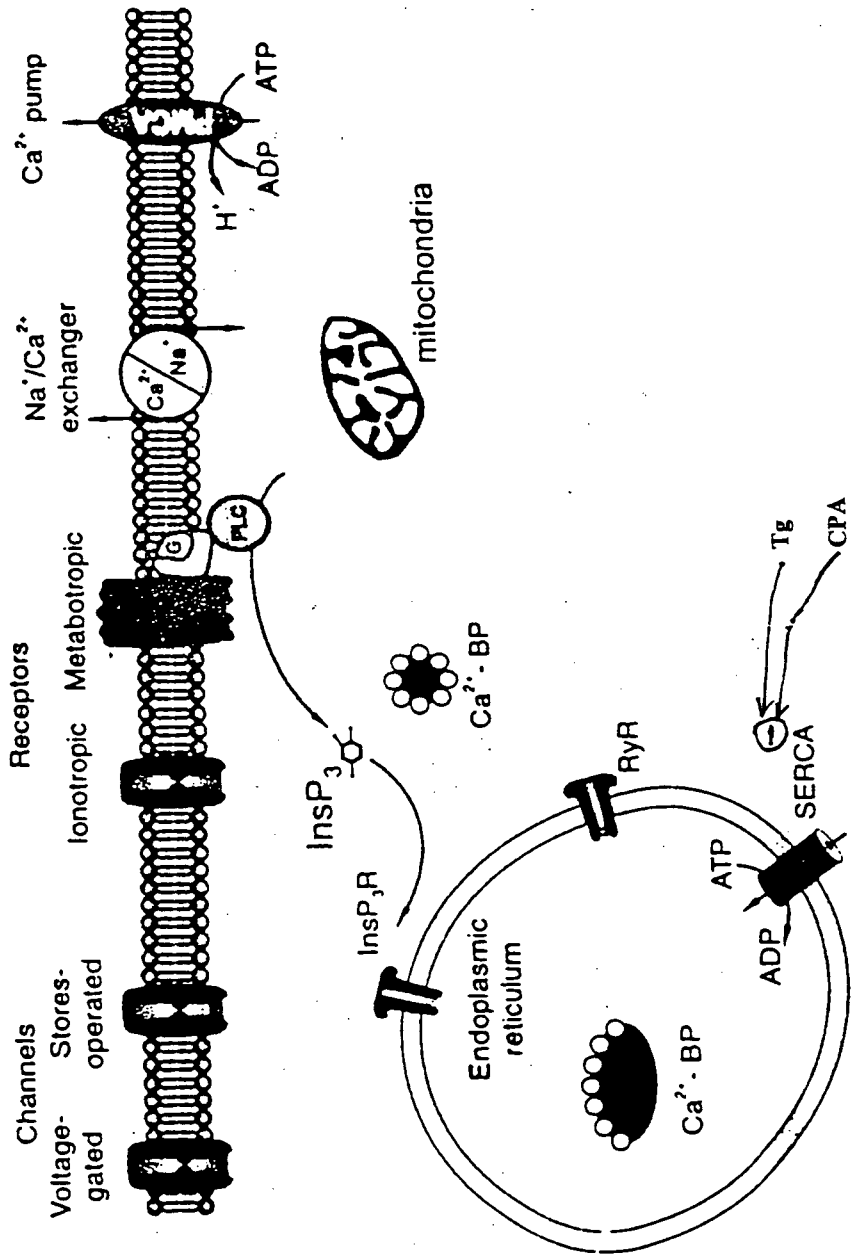
express scavenger receptors that may play a significant role in the clearance of Aβ plaques. Both the contribution of microglia and Aβ to the etiology of AD remains undefined. Thus, it will be very important to define the events regulating microglial activity.

2. Calcium entry into cells

In a wide variety of cell types, cell function is regulated by changes in the cytosolic free calcium ion concentration ($[Ca^{2+}]_i$). Ca^{2+} serves as an important second messenger in cell regulation to link electrical or chemical excitation and cellular responses. $[Ca^{2+}]_i$ can undergo large and rapid increases (up to 100-fold) following the binding of agonists to their cell surface receptors. The extracellular ionized calcium concentration ($[Ca^{2+}]_o$) is about 1.3 – 1.8 mM, about 10,000-fold higher than the basal level of $[Ca^{2+}]_i$, which is typically in the range of 50 – 200 nM in the majority of cells and therefore presents a virtually inexhaustible source for $[Ca^{2+}]_i$. The steep concentration gradient between the intra- and extracellular space is maintained largely by two surface membrane proteins, a Na^+/Ca^{2+} exchanger protein and an ATP-dependent Ca^{2+} pump, Ca^{2+}-ATPase (PMCA), as well as a distinct intracellular Ca^{2+}-ATPase (SERCA) on sarcoplasmic reticulum (SR) or endoplasmic reticulum (ER), which sequesters Ca^{2+} into the intracellular storage pool ER or SR (Blaustein et al., 1991).

Several pathways exist for Ca^{2+} entry into cells, some of them are shown in schematic diagram 1. An increase of $[Ca^{2+}]_i$ can follow two main routes which include calcium influx into cells through the plasma membrane, and release of calcium from intracellular stores (like ER and SR).

2.1 Voltage-dependent Ca^{2+} channels



Schematic diagram 1 Pathways for Ca^{2+} entry into cytosol. Ca^{2+} entry from the extracellular space can occur through voltage-gated calcium channels (VDCC), store-operated calcium channels (SOC) / capacitative Ca^{2+} entry, ionotropic receptor-operated Ca^{2+} channels and metabotropic receptor-operated Ca^{2+} channels. Ca^{2+} can also enter the cytosol from internal stores through the PLC-IP_3 pathway. SERCA is responsible for Ca^{2+} sequestration into stores which can be inhibited by Tg (tharpsigargin) and CPA (cyclopiazonic acid).

Ca^{2+} influx into the cell occurs through voltage-dependent Ca^{2+} channels (VDCC). The VDCC comprise a group of multi-subunit membrane spanning proteins which form a micropore and allow Ca^{2+} influx when activated by membrane depolarization. They have been divided into different subtypes like the L-, N-, T-, P-, and Q-type channels based upon their pharmacological sensitivities and activation characteristics that result in different patterns of Ca^{2+} influx between the different cell types (Snutch et al., 1990; Swandulla et al., 1991).

2.2 Receptor-operated Ca^{2+} channels

Like VDCC, receptor-operated Ca^{2+} channels (ROC) are also multimeric proteins which span the plasma membrane or intracellular membranes. Ca^{2+} influx by ROC follows activation of the receptor by the binding of a specific ligand or agonist to the receptor complex to activate the related ion channels. Ca^{2+} entry through ROC activation may take several forms (Sage, 1992). A ROC may be formed by subunits of the multimeric receptor protein, or be closely linked to the ion channel receptor on the membrane via a GTP-binding protein.

2.3 Intracellular store release of Ca^{2+}

Release of calcium from intracellular stores is another important way of increasing $[Ca^{2+}]_i$ in either excitable or non-excitable cells. The major signalling mechanism responsible for this pathway is the phospholipase C (PLC)-inositol phosphate (IP) signalling system (Berridge, 1993). In response to many stimuli (such as neurotransmitters, hormones and growth factors), either G protein-linked receptors, or other receptors linked directly or indirectly to tyrosine kinases are activated. The activation of these receptors are coupled to energy-requiring (GTP or ATP) transducing mechanisms which activate PLC to hydrolyse the inositol lipid

precursor, phosphatidylinositol 4,5-biphosphate stored in the plasma membrane, to give both diacylglycerol (DAG) and inositol triphosphate (InsP_3). The latter then acts as a diffusible second messenger and binds to an InsP_3 receptor (InsP_3R) on the internal storage membrane system. Upon activation, the InsP_3R undergoes a conformational change which is perhaps related to the coupling process leading to Ca^{2+} channel opening (Mignery and Sudhof, 1990). Ca^{2+} released from the storage sites increases $[Ca^{2+}]_i$.

Another major family of intracellular Ca^{2+} channels mobilizing stored Ca^{2+} are ryanodine receptors (RYRs) which have three family members: the skeletal RYR1, cardiac RYR2 and RYR3 in some non-muscle cells (Berridge, 1993). Cells having RYRs are sensitive to ryanodine and caffeine signals.

The distribution of InsP_3- or ryanodine-sensitive Ca^{2+} stores varies considerably from cell to cell. Some cells either have ryanodine-sensitive stores (mainly skeletal muscle) or InsP_3-sensitive stores, whereas other cells contain both. There is considerable structural and molecular homology shared by InsP_3R and RYR, thus they have some functional similarities (Henzi and MacDermott, 1992). For example, a small influx of Ca^{2+} through VDCC or ROC triggers an explosive release of Ca^{2+} from the SR in heart cells (Friel & Tsein, 1992). This positive feedback effect produces a regenerative process called calcium-induced calcium release (CICR), which is a property of RYRs and may also be displayed by InsP_3.

Ca^{2+} stored in intracellular ER or SR is sequestered by Ca^{2+}-ATPases located on the membrane surface of SR/ER (SERCA) which pump the cytosolic Ca^{2+} into the stores. Tharpsigargin (Tg), and cyclopiazonic acid (CPA) are well known Ca^{2+}-ATPase inhibitors that can block the SERCA pump and thus empty the internal Ca^{2+} stores.

2.4 Capacitative Ca^{2+} entry.

It has been found that Ca^{2+} release from intracellular stores is regulated and functionally coupled with Ca^{2+} influx (Berridge, 1995). This means that when internal Ca^{2+} stores are depleted, Ca^{2+} influx occurs through the plasma membrane. This idea was first introduced by Putney, who hypothesised that Ca^{2+} release from internal store is coupled, in some way, to the activation of Ca^{2+} entry, termed capacitative Ca^{2+} entry (Putney, 1986). Later, considerable evidence showed that this internal Ca^{2+} store-regulated capacitative Ca^{2+} entry is present in many cell types and has properties which are ubiquitous (reviewed by Putney, 1990).

Capacitative Ca^{2+} entry can be produced by a large variety of stimuli such as agonists or pharmacological agents, all of which share the common property of releasing stored calcium. Examples include Ca^{2+}-mobilizing agonists such as PKC, Ca^{2+}, 5-hydroxytryptamine (5-HT) (Parekh et al., 1993; Petersen and Berridge, 1994) , the Ca^{2+}-mobilizing second messenger, $InsP_3$ (Petersen and Berridge, 1994; Hoth and Penner, 1992), the calcium ionophore ionomycin (Hoth and Penner, 1992; Morgan and Jacob, 1994), inhibitors of the ER Ca^{2+}-ATPase pumps such as thapsigargin (Zwerfach and Lewis, 1993) and cyclopiazonic acid (Mason et al., 1991), or simply by incubating cells in Ca^{2+}-free conditions (Hoth and Penner, 1992; 1993). All of this evidence indicates that the entry of external calcium is somehow controlled by the calcium content of the intracellular stores.

Although capacitative Ca^{2+} entry appears to be frequent, the means by which Ca^{2+} store depletion leads to Ca^{2+} entry across the plasma membrane, and how information is transmitted across the gap separating the ER from the plasma membrane is not understood. To explain the capacitative mechanism, two models have been proposed. One is the Ca^{2+}-influx factor (CIF) model which proposes the existence of a soluble CIF, which is released after Ca^{2+} stores are

depleted, and stimulates Ca^{2+} influx via Ca^{2+} channels on the plasma membrane (Randriamapita and Tsien, 1993; Thomas et al., 1996; Bird et al., 1995). There are indications that protein phosphatases may regulate the responsiveness of Ca^{2+} entry channel to CIF, implying that the latter may act by promoting channel phosphorylation (Randriamampita and Tsien, 1995). In this respect, phosphatase inhibitors are also able to enhance the responsiveness of cells to threshold levels of either CIF or Ca^{2+}-mobilizing agents like thapsigargin and carbachol.

Another hypothesis for the mechanism of capacitative Ca^{2+} influx is the conformational coupling (protein-protein interaction) model. This model postulates that internal Ca^{2+} release channels ($InsP_3Rs$) on the store membrane function as a 'go-between' of two membrane systems by conformational coupling to store-depletion operated Ca^{2+} channels on the plasma membrane (Irvine, 1990; Berridge, 1995). By this coupling, a mechanical link is formed from the store $InsP_3R$ to the channel in the plasma membrane. The signals that regulate capacitative Ca^{2+} entry are integrated by the large cytoplasmic head of the $InsP_3$ receptor which then transmits the information directly to calcium entry channel through a protein-protein interaction.

2.5 Extracellular Ca^{2+}-sensing receptor

Among ROCs, is an important extracellular Ca^{2+}-sensing receptor (CaR) which has been demonstrated in many tissues in recent years. This CaR is present in parathyroid gland, thyroid C-cells, placenta, keratinocytes, kidney and brain (Brown et al., 1995). The CaR is responsive to many polyvalent cations such as Ca^{2+}, Mg^{2+}, Gd^{3+} and neomycin with the order of potency as Gd^{3+}>neomycin>Ca^{2+}>Mg^{2+}. This receptor was thought to be the first example of a G-protein-coupled cell-surface receptor in a mammalian species which recognises inorganic ions, as opposed to organic ligands (Conklin et al., 1994; Taylor, 1994; Clapham, 1993). Through the G

protein coupled CaR, increases in $[Ca^{2+}]_o$ activity can modulate the production of two major intracellular second messenger systems (Brown et al., 1991; Jackson, 1991). First, raising $[Ca^{2+}]_o$ activates PLC, leading to accumulation of $InsP_3$ (Brown et al., 1987) and release of Ca^{2+} from intracellular calcium stores (Nemeth et al., 1990 & 1987). The rise in $[Ca^{2+}]_i$ is sustained by an accompanying influx of Ca^{2+} through Ca^{2+} channels in the plasma membrane (Nemeth, 1990). In addition, the increased $[Ca^{2+}]_o$ is capable of inhibiting a receptor-mediated (e.g. dopamine-mediated) increase in cAMP. The latter effect can be blocked by pre-treatment with pertussis toxin implicating a G_i-like protein in the transmission of the $[Ca^{2+}]_o$ signal. These changes in second messengers may modulate the activity of a series of kinases, such as PKC and PKA, that, in turn, could alter the biological activity of the cell.

In brain, CaR transcripts have been detected in many regions (hypothalamus > corpus striatum > hippocampus, cortex, cerebellum, brainstem and pituitary) (Ruat et al., 1995). CaR protein was found throughout the brain by immunohistochemical staining with CaR antibody. This intracerebral localization is consistent with an association of the CaR with nerve terminals. It is possible that local changes in $[Ca^{2+}]_o$ provides a local feedback signal transduced by the Ca^{2+}-sensing receptor which is involved in regulating brain function.

2.6 Calcium changes in microglia

The transformation of resting microglia into intrinsic brain macrophages is likely a multistep process which appears to be under strict control. The transition of macrophage phagocytosic function and the release of oxygen radicals by macrophages has been reported to be preceded by an increase in $[Ca^{2+}]_i$. A rise in the level of $[Ca^{2+}]_i$ has also been shown to be a mediator of the cellular response of peritoneal macrophages and other immune cells (Letari et

al., 1991). By analogy with this, $[Ca^{2+}]_i$ is thought to play a role in microglial activation. Recently, a number of studies have investigated the $[Ca^{2+}]_i$ changes in cultured microglia with different stimuli. For example, $[Ca^{2+}]_i$ has been observed to be increased in rat or mouse microglia by exposure to a variety of agents, including LPS, a potent activator of the immune system (Bader et al., 1994), and endothelins, potent_vasoconstrictors (Moller et al., 1997), extracellular ATP (Walz et al., 1993, Ferrari et al., 1996), carbachol (CCh) (Whittemore et al., 1993; Tanabe et al., 1997), inflammatory mediators like chemokine and complement C_5 (Nolte et al., 1996; Tanabe et al., 1997), platelet-activating factor (PAF) (Mori et al., 1996), human prion protein (PrP) which are toxic to cultured neurons (Herms et al., 1997) and beta-amyloid 25-35, a synthetic fragment of APP (Korotzer *et al,* 1995). Phorbol ester, a general activator of PKC, has also been reported to increase the $[Ca^{2+}]_i$ in cultured human microglia (Yoo et al., 1996). Thus, increased $[Ca^{2+}]_i$ in microglia, which is induced by different agonists might be involved in the functional transformation of microglia and their cytotoxic activity. In fact, it has been shown that superoxide production in microglia is induced by an increase of $[Ca^{2+}]_i$ (Colton et al., 1995).

3. Neurotransmitter receptors on microglia

3.1 Effects of ATP on microglia

ATP is released in the CNS as a co-transmitter (with noradrenaline, or acetylcholine) from neurons and endothelial cells (Burnstock 1990). This nucleotide accumulates with acetylcholine and noradrenaline in cholinergic and adrenergic nerve terminals respectively (Zimmerman, 1994); furthermore, specific ATP-containing terminals have also been described (purinergic neurons) (Burnstock, 1972). In addition, ischemic, injured and dying cells release ATP in large amounts (Forrester and Williams, 1997). It is possible that tissue injury will

release ATP from cells and might act as an initial trigger of an inflammatory or regenerative process in the CNS, where microglia could mediate part of this effect. Extracellular ATP exerts its effects through purinergic receptors (termed 'purinoceptors'). Basically, two types of purinoceptors have been postulated, one type responds to adenosine (P_1) and the other type responds to ATP (P_2). Studies of the purinoceptors of macrophages and microglia have suggested that P_2 purinoceptors should be placed into two major families on the basis of their unique transductional characteristics: a P_{2x} ionotropic purinoceptor family consisting of ligand-gated cation channels and a P_{2y} metabotropic purinoceptor family consisting of GTP-protein-coupled receptors (Fredholm et al., 1994). Other P_2 purinoceptors subtypes have been reported like P_{2U}, which is also coupled to a G protein but is activated both by ATP and the pyrimidine nucleotide uridine 5'-triphosphate (UTP). P_{2z} is another purinoceptor which was first found in macrophages and lymphocytes and responds selectively to tetra-anionic ATP (Gordon et al., 1987). Activation of the P_{2z} receptor on the plasma membrane resulted in the appearance of 800KDa pores which permit the passage of inorganic cations as well as the uptake of extracellular markers such as ethidium bromide and lucifer yellow (Steinberg et al., 1987). Recently, the P_{2z} receptor has been identified as belonging to the growing family of ionotropic P_{2x} receptors (Surprenant et al., 1996), and has consequently been termed P_{2x7}. The presence of ATP-sensitive P_{2x}, P_{2y} and P_{2z}/P_{2x7} purinoceptors have been demonstrated in cultured rat microglia (Norenberg et al., 1994b; Ferrari et al., 1996).

There is evidence that extracellular ATP produces biological actions like cell proliferation and differentiation, activation of the respiratory burst and cell cytotoxicity in the immune system through the involvement of increases in $[Ca^{2+}]_i$. These Ca^{2+} responses, triggered by extracellular ATP in the immune system, might be due to the action of different plasma P_2

receptor subtypes. Exposure of macrophages and lymphocytes to ATP can stimulate a large increase in $[Ca^{2+}]_i$ (Steinberg 1987). Thus, in analogy with macrophages, extracellular ATP has also been regarded as a candidate activator for microglia.

The effects of ATP on $[Ca^{2+}]_i$ in both cultured mouse microglia and microglial cell lines has been documented (Walz et al., 1993; Ferrari et al., 1996). The dose-response effects of ATP on cultured mouse microglia showed that the threshold of the $[Ca^{2+}]_i$ increase in response to ATP occurred at a concentration around 10^{-6} M, and the maximal response was reached at 10^{-5} M (Walz et al., 1993). This $[Ca^{2+}]_i$ increase was rapid, with rise times of 10-16 sec and recovery over about 1 min. Application of ATP (<0.1 mM) and its analogue 2' –and 3' –o- (4-benzoylbenzoyl) -ATP to microglial cell line N9 and N13 activates a spiking Ca^{2+} release from intracellular stores as well as a massive transmembrane Ca^{2+} influx (Ferrari et al., 1996). In this case, the ATP-stimulated large Ca^{2+} spike was followed by a long lasting plateau, which was suggested to be due to sustained Ca^{2+} influx across the plasma membrane, since removal of external Ca^{2+} fully abolished the plateau. These results suggested that mouse microglia posses two P_2 receptor subtypes with possibly different affinities for extracellular ATP: one, P_{2y}, coupled to the release of stored Ca^{2+} occurs due to the formation of $InsP_3$ through the activation of PLC pathway; the other, P_{2x}, coupled to transmembrane ion flux (Ferrari et al., 1996). This result closely agreed with studies in J774 macrophage cell lines, where two distinct nucleotide-mediated Ca^{2+} flux mechanisms were postulated (Greenberg et al., 1988).

The study of Ferrari et al (1996) also showed that microglia had an uptake of extracellular markers when stimulated by high concentrations of ATP (1 mM) which suggested the existence of P_{2z} /P_{2x7} receptors on these cells. One effect of the dose-dependence of the P_2 receptor subtypes was that at low concentrations of extracellular ATP only P_{2y} and P_{2x} receptors

would be activated, and that could occur in a wide range of target cells, but at high concentrations, ATP would also activate P_{2Z}/P_{2X7} receptors in microglia leading to a distinct cell response (Ferrari et al., 1996).

3.2 Effects of cholinergic agonists on $[Ca^{2+}]_i$ in cultured microglia

Acetylcholine is a major excitatory neurotransmitter in the mammalian CNS. Cholinergic neurotransmission is mediated by two classes of receptor, the G-protein coupled muscarinic receptors and the ligand-gated nicotinic receptors. Cholinergic transmission at muscarinic acetylcholine receptors (mAChR) has been implicated in higher brain functions such as in learning and memory, and a loss of cholinergic synapses may contribute to the symptoms of AD. Many studies have examined mAChR subtypes because this family of receptors is involved in central cholinergic transmission. Pharmacologically distinct forms of the mAChR have been classified into muscarinic M1, M2 and M3 receptors according to the selectivity of novel antagonists. All of the subtypes appear to be present in brain, however, are differently distributed. Immunoprecipitation studies have shown close agreement between the distribution of mAChR subtypes in of rat (Yasuda et al., 1993) and human brain (Flynn et al., 1995).

It has been demonstrated that phagocytic leukocytes have stereo-selective muscarinic receptors (Lopker et al., 1980), and that carbachol could induce polarization and chemotaxis of these cells (Stephens and Snyderman, 1982). When eosinophilic leukemia cell line-1 are treated with INF-γ, M3 and M5 receptor mRNAs could be detected along with an increase in $[Ca^{2+}]_i$ and chemotaxis induced by carbachol (Mita et al., 1996). This supports the functional importance of mAChRs in monocytic/macrophagic cell differentiation.

The response of microglia to neurotransmitters suggested that there may exist a signaling loop between neurons and microglia. This signal could arise from neuronal activity, or a microglial response to brain injury or tissue damage. Interestingly, the M1, M3 and M5 receptors also selectively influence the processing of the APP, such that mAChR activation increases the secretion of non-amyloidogenic peptides (Faber et al., 1995). In addition, microglial processes have been reported to be associated with synapses anatomically (Pow et al., 1989). Specifically, cholinergic fibers have been shown to exhibit reactive synaptogenesis in the hippocampus in AD, and cholinergic fibers are known to infiltrate neuritic plaques (Selkoe, 1991; Strubble et al., 1982). Previous studies have shown that cultured rat microglia respond to carbachol exposure, a nonspecific muscarinic agonist, with an increase in $[Ca^{2+}]_i$ which is atropine sensitive, suggesting that rat microglia have functional muscarinic receptors (Whittemore et al., 1993). Muscarinic receptors are G protein-coupled seven-transmembrane-spaning receptors usually associated with pertussis toxin-resistant G proteins (G_i or G_o) (Dohlman et al., 1991). For example, mAChR stimulation is linked through mainly G_i protein to various cardiac functional effects including activation of potassium channels and inhibition of voltage-dependent calcium channels (Hescheler et al., 1986). But carbachol-induced $[Ca^{2+}]_i$ increase in microglia is not affected by pertussis toxin treatment (Tanabe et al., 1997), indicating that mAChR activation-induced $[Ca^{2+}]_i$ increase in microglia is not through the G_i protein coupled calcium channels; instead, it may through the PLC-$InsP_3$ pathway like some other cell types. For instance, studies of the ACh-induced $[Ca^{2+}]_i$ increase in endothelial cells showed that muscarinic receptor activation turned on the PLC-$InsP_3$ pathway, triggering internal Ca^{2+} store release followed by Ca^{2+} influx (Wang et al., 1995).

As there is expression of muscarinic cholinergic receptors in rat microglia, it has been hypothesized that abnormal cholinergic inputs in the senile plaques of AD tissue result in the activation of microglia via muscarinic receptor activation. In turn, this produces an increase in $[Ca^{2+}]_i$, which then begins an inappropriate or abnormal response to damage healthy tissue (Whittemore et al., 1993).

4. Electrophysiological properties of microglia

The rapid responses of microglia may be associated with changes in membrane properties, since a significant function of microglia in brain is to interact with surrounding cells. In contrast to peripheral macrophages, which possess voltage-dependent inwardly and outwardly rectifying potassium conductances (Gallin and Sheehy, 1985), most functional microglia (ameboid form) exhibit a different ion channel pattern, characterized by a large, inwardly rectifying potassium current and no outward currents (Kettenmann et al., 1993). The unique membrane current pattern of microglia has been described both in cell culture (Kettenmann et al., 1993) and in intact brain tissue (Brockhaus et al., 1993). The finding of a prominent inwardly rectifying potassium conductance in microglia is shared by a sub-population of bone marrow-derived cells which may be macrophage-precursors with a lineage relationship to brain microglia (Banati et al., 1991). This supports the hypothesis that the differentiation of monocytes into microglia occurs during late embryonic development.

Extracellular Cs^+ and Ba^{2+}, two blockers of inwardly rectifying potassium channels nearly abolish the inward conductance in microglia. Inward K^+ current is the most consistently expressed current in microglia, being found in most cultured rat and mouse microglia (Banati et al., 1991; Bocchini et al., 1992; Brockhaus et al., 1993; Fischer et al., 1995; Kettenmann et al.,

1990, 1993; Korotzer and Cotman, 1992; Norenberg et al., 1992, 1993, 1994a; Walz et al., 1993), and in bovine microglia (McLarnon et al., 1995). In human microglia, small inward K^+ currents are also observed in early adherent cells (1 –2 days after cell plating) (McLarnon et al., 1997). However, inward K^+ current was not seen in another report on human microglia (Norenberg et al., 1994c). The outward K^+ current can rarely be observed in unstimulated microglia (Kettenmann et al., 1993). Incubating rat microglia with LPS (100ng/ml) led to the expression of an additional outward current in response to membrane depolarization, in addition to the previously existing inwardly rectifying K^+ channels (Norenberg et al., 1992). Outward rectifying K^+ channels have also been documented in human microglia following exposure to INF-γ (McLarnon et al., 1997) and in murine microglia following exposure to granulocyte-macrophages-colony-stimulating factor (GM-CSF) (Fischer et al., 1995), chemotactic agent complement C5a and epidermal growth factor (Ilschner et al., 1996). The electrophysiological responses of unstimulated microglia are dominated by an inward rectifying K^+ conductance. Based on the observations described above, the appearance of outward-rectifying K^+ conductance is now seen as a sign of microglial activation.

Since complement C_5 can also induce transient $[Ca^{2+}]_i$ elevation (Nolte et al., 1996), an interesting question is if the induction of the K^+ conductance is linked to this $[Ca^{2+}]_i$ response. Ilschner et al (1996) showed the K^+ conductance was not activated when a Ca^{2+} transient was induced by thapsigargin, nor did blockade of the C5a-induced K^+ conductance by K^+ channel blocker affect the $[Ca^{2+}]_i$ response, suggesting that following activation of C5a receptor, expression of the outward K^+ conductance and Ca^{2+} mobilization are governed by independent intracellular pathways. But there have been reports of a Ca^{2+}-dependent K^+ channel in microglia (Langosch et al., 1994; McLarnon et al., 1995, 1997).

Effects of ATP on ion channels in microglia

Exposure of macrophages and lymphocytes to ATP has been shown to stimulate transmembrane ion fluxes and membrane depolarization (Steinberg and Silverstein, 1987). Patch-clamp studies demonstrated that this interaction is sometimes via ATP stimulation through a P_{2z}/P_{2x7} receptor (Buisman et al., 1988). Studies on the effects of ATP on electrophysiological properties of mouse microglia *in vitro* have suggested that ATP (less than 100 μM) can act on P_{2x} purinoreceptors to transiently activate a rapidly desensitizing inward current at a holding potential of –70 mV. This is due to the opening of non-selective cationic channels with permeability to inward Na^+ and Ca^{2+} and outward K^+ (Kettenmann et al., 1993; Walz et al., 1993; Norenberg W., 1994b). This ATP-activated inward current in mouse microglia disappears with repetitive application of ATP. The ATP-activated opening of pores in the plasma membrane allows not only the free passage of inorganic cations and anions, but also the entry of fluorescent dyes (Ferrari et al., 1996). This finding is in agreement with that observed in macrophages (Steinberg et al., 1987), suggesting that microglia possess P_{2x} receptors. The activation of this membrane conductance in macrophages and microglia might be due either to the new synthesis of channel proteins or the activation of pre-existing silent channels.

As with other activators of microglia, application of ATP to microglia produces an outward K^+ current (Ilschner et al., 1995,1996; Norenberg et al., 1994b; Walz et al., 1993). Recently, ATP-induced membrane currents have also been recorded from microglia acutely isolated from a mouse corpus callosum brain slice preparation, indicating that several subtypes of purinoceptors are present (Haas et al., 1996).

5. Properties of human and rodent microglia

From the previous literature, we know that human microglia differ from microglia derived from other species in a number of properties. For example, NO production occurs in rodent rat microglia (Boje and Arora, 1992; Merrill et al., 1993), but not in human microglia (Walker et al., 1995). Treatment of human adult microglia with LPS and Aβ does not induce the production of NO or the expression of iNOS mRNA (Walker et al., 1995). This observation was also consistent with the report that human fetal microglia do not produce NO (Lee SC et al., 1993). In addition, the capacity of rodent microglia to proliferate in response to CSF-1 is different from microglia isolated from human adult brain (Williams et al., 1992). These observations indicate that the properties of microglia are subject to species variation.

6. Objectives:

Clearly, it is important to determine whether Ca^{2+} is involved in microglial function or to the pathogenesis of neurodegenerative diseases. Such possibilities cannot be explored only through the evaluation of rodent microglia. Thus, studies of human microglia are of importance to understand human neurodegenerative diseases like AD.

Although we have some understanding of $[Ca^{2+}]_i$ changes in rodent microglia in response to ATP or cholinergic agonists, little is known about their effects in human microglia. Since microglia are associated with AD tissue, and calcium has long been established as an important second messenger in the CNS, experiments on cultured microglia, using a Ca^{2+}-indicators such as fura-2, would be helpful in investigating the relationship between Ca^{2+} and putative stimulators in human microglia. My hypothesis is that cultured human microglia will show changes in $[Ca^{2+}]_i$ in response to two agonists which are present in the CNS, namely ATP and

ACh. These stimuli may also modify the properties of human microglia and may be relevant to human neurodegenerative diseases. The basis of the is to study Ca^{2+} regulation in microglia using primary cultures of fetal human brain and to investigate the responses of these cells to ATP, cholinergic agonists such as CCh and ACh, and extracellular free Ca^{2+} concentration. In addition, the electrophysiological properties of ATP on human microglia are still poorly understood. Using whole-cell patch-clamp technique, the effects of ATP on microglial ion channel expression were examined.

Chapter two MATERIAL AND METHODS

1. Primary cell culture

Human microglial cultures were prepared from fetal human brains as previously described (Kim et al., 1983; Satoh and Kim, 1995). Fetal human brain tissue (12-20 weeks gestation) were obtained following therapeutic abortion (legal abortions were performed in an authorised centre and approval for the use of tissues was obtained from the Ethics Committee of the University of British Columbia). After removal of the meninges, brain tissues were dissected into small pieces, then incubated in Dulbecco's phosphate-buffered saline (PBS) containing 0.25% trypsin and 40 μg/ml DNase for 30 min at 37°C, and dispersed into single cells by gentle pipetting. Cells were collected by centrifugation at 2400 rpm for 20 min. Dissociated cells were suspended in feeding medium consisting of Dulbecco's modified Eagles' medium containing 5% horse serum, 5 μg/mg glucose and 25 μg/ml gentamicin, plated in 75 cm^2 culture flasks at a density of 10^6 cells/ml, and incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air. Media was changed twice a week. After one to two weeks, cells grown in flasks consisted of neurons, oligodendrocytes lying on top of an astrocyte layer and free-floating microglia. Microglia-enriched cultures were obtained by collecting the floating cells; the microglia were then plated on 25 mm round glass coverslips for fluorescence imaging studies. All the cultured microglia cells were kindly provided by Dr. Kim's lab in the Neurology Division, Department of Medicine, University of British Columbia, Vancouver.

2. Fluorescence studies

Fluorescence imaging using fluorescent dyes was performed to measure the $[Ca^{2+}]_i$. The fluorescent Ca^{2+} indicator, fura-2, is widely used for the measurement of $[Ca^{2+}]_i$. A property of

fura-2 is that when it is loaded into the cell, the dye has two molecular forms, free and Ca^{2+}-bound. Both the free form of fura-2 and its Ca^{2+} complexed form fluoresce strongly, but their excitation peaks differ in wavelength. Fura-2 shifts its excitation peak from about 380 nm to 340 nm upon binding Ca^{2+}. The extent of the shift between the two wavelength depends on the amount of $[Ca^{2+}]_i$.

2.1 Cell loading with fura-2

Microglial cells grown on 25-mm round glass coverslips were incubated at 37°C for 25 min in loading buffer. The loading buffer included: the fluorescent dye fura-2 acetoxymethyl ester (fura-2/AM; Molecular Probes, Eugene, OR), dissolved in dimethyl sulfoxide (DMSO) as a 1 M stock solution, made into final concentration of 5 μM, plus 0.03% pluronic acid (Molecular Probes) and 0.02 % bovine serum albumin (BSA), in a HEPES-buffered Hanks' balanced salt solution (HBSS, pH 7.4), containing (in mM) NaCl, 145; KCl, 2.5; $MgCl_2$, 1.0; $CaCl_2$, 1.8; HEPES, 20; glucose, 10. The final concentration of DMSO in the loading buffer did not exceed 0.2%. The calcium-free experiment utilised the same buffer but $CaCl_2$ was omitted and 50 μM EGTA was added. This buffer supplemented with glucose can increase the cell viability over extended periods. In experiments using elevated external K^+, 50 mM KCl was used to replace an equivalent amount of NaCl. Fura-2 was loaded into the cells by incubating the microglia with the membrane-permeant ester derivatives. When cytosolic esterases cleave the ester group from these compounds, the membrane-impermeant dye is trapped within the cell. An increase in $[Ca^{2+}]_i$ increases the amount of Ca^{2+} binding to the dye and thus the intensity of fluorescence is increased proportionately.

2.2 De-esterification

Since the incomplete de-esterification of the dye in cytoplasma will interfere with the $[Ca^{2+}]_i$ measurement, cells loaded with fura-2 should be de-esterified completely. The microglia on glass coverslips were washed twice with HBSS and allowed to incubate in the same buffer for at least an additional 30 min to ensure complete de-esterification of fura-2/AM.

2.3 Determination of $[Ca^{2+}]_i$

The coverslip was then transferred to a perfusion chamber with a working volume of 0.3 ml on a fluorescence imaging system, and usually 5-10 cells were chosen for recording on each coverslip. The fluorescent image was measured using a Nikon 40X fluorite epifluorescence objective fitted to an inverted microscope, equipped with a 100 Watt xenon light source(Osram, Germany). During imaging, light was selectively filtered at 340 and 380 nm by a computer controlled filterwheel. Emitted fluorescence was detected by a silicone intensified target (SIT) video camera and fed to an 80386 based desktop computer equipped with image-detection software (Image-I, Universal Imaging) and an analog-to-digital video digitizer board. The software also controlled the shutter/filter-wheel and displayed images taken at two wavelength after background subtraction, as well as an image of 340/380 nm ratio on the monitor (fura-2 was excited at a wavelength of 340 nm and 380 nm). The intensity ratios (340nm/380nm) were than converted into $[Ca^{2+}]_i$ by using the method of Grynkiewicz et al. (Grynkiewicz et al., 1985):

$$[Ca^{2+}]_i = K_d \times \beta \times (R - R_{min})/(R_{max} - R)$$

In this equation, K_d is the dissociation constant of the Ca^{2+}-fura-2 complex, which is determined at 25°C (around room temperature) according to this formula by using a series of calibration buffers by using 5 μM fura-2 free acid with known amounts of free Ca^{2+}. All the cell recording performed in this study is under the room temperature; R is the ratio of fluorescence intensity due to excitation

at 340 nm and 380 nm; R_{max} is the maximum value of R when all of fura-2 is saturated with Ca^{2+}, which is measured by adding 4-Bromo A23187 (10 μM) to a 5 mM Ca^{2+}- containing HBSS; R_{min} is the minimum value of R when all of fura-2 is in the Ca^{2+}-free form, which is measured by adding EGTA 10 mM (pH 8.0) to Ca^{2+}-free HBSS; β is the ratio of 380 nm signals in Ca^{2+}-free and Ca^{2+} saturate solution (S_{f380}/S_{b380}). One of the fura-2 in vivo calibrations is shown in Fig. 1. All the data was analysed using a spreadsheet (MS Excel) and plotted. Data is shown as the mean ± SEM, and unpaired T test used for statistics.

3. Patch-clamp recording

The membrane currents of cultured microglia was recorded using the patch-clamp technique in the whole-cell configuration. In this method, a glass micropipette is used to contact the surface of the cell. Because the diameter of the pipette tip is in the range of 1-5 μm, the contact between the tip of the pipette and the cell membrane electrically isolates the small patch of membrane within the tip. Gentle suction to the pipette induces an extremely tight seal (a gigaohm seal) between the pipette orifice and the lipid bilayer. A whole-cell recording mode is then obtained by suction applied to the pipette or using an electrical pulse from the amplifier. The recording bath was grounded, with the electrode separated from bath solution by a short agar bridge to enable the potential inside the pipette to be clamped at the desired level.

The methods used in whole-cell recordings from human and bovine microglia have been previously documented (McLarnon et al., 1997; McLarnon et al., 1995). Briefly, cultured human microglia plated on 9 mm plastic coverslips were placed on the stage of an inverted microscope (Nikon TMS) and whole-cell macroscopic currents were recorded using an Axopatch 200A amplifier (Axon Instruments, Burlingame, CA). Patch pipettes were pulled

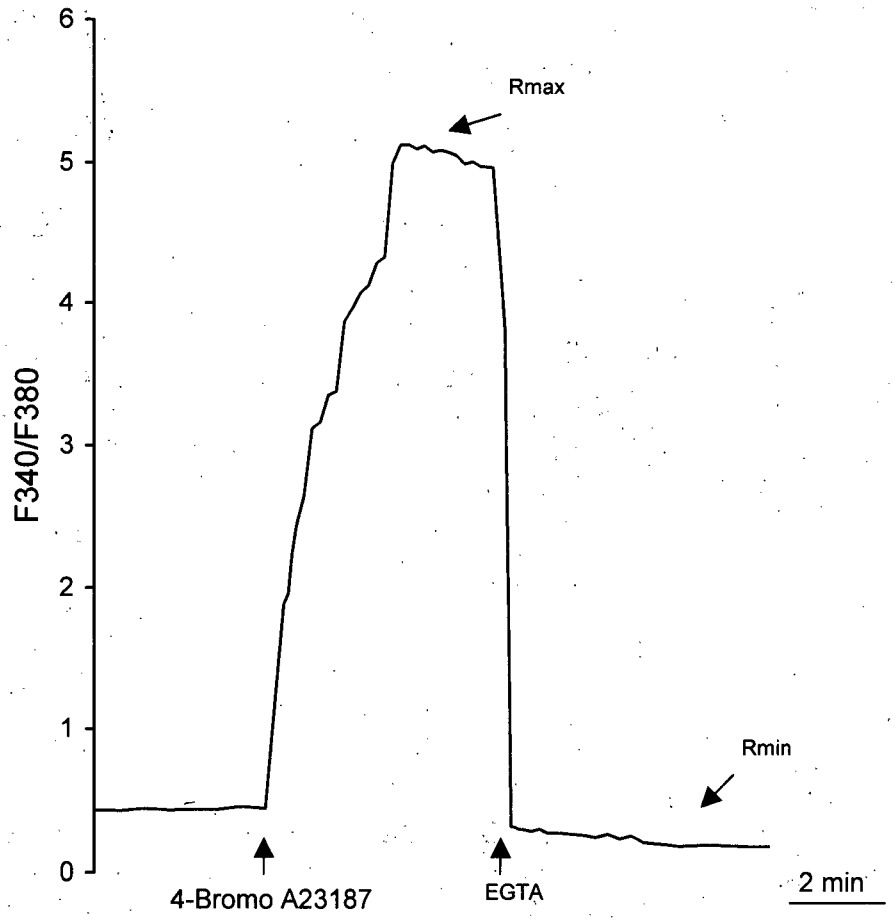


Fig. 1 Fura-2 in vivo calibration in human microglia. 4-Bromo-A23187 (10 uM) in 5 mM Ca^{2+}-containing HBSS was used for the Rmax value; EGTA (10 mM, pH 8.0) in Ca^{2+}-free HBSS was used for the recording of Rmin value. The base line, Rmax and Rmin value were 0.43, 5.12 and 0.17 respectively.

from Corning Glass # 7052 with a micropipette puller. When filled with intracellular solution (pipette solution), the pipette has a resistance in the range of 2-4 MΩ. During whole-cell recordings the series resistance was compensated by 85-90% using the series resistance-compensation of the amplifier; leak subtraction was also used on occasion. Application of stimuli to the cell (ATP 0.1 mM) is through a gravity-fed extracellular perfusion system, which was constructed using syringes. The experimental set-up of these solutions was made in such a way that their height could easily be adjusted.

A holding potential of –60 mV was used in all experiments and voltage-clamp protocols were generated by computer. The protocols included a series of depolarizing steps to activate outward K^+ currents; in most experiments single steps to +40 or +60 mV were used. In several experiments procedures were employed to determine the reversal potentials of tail currents following depolarizating pulses. A depolarization step is first applied (to +40 mV) to activate outward current followed by test potentials from 0mV to –100 mV. The current at the beginning of the test step was used as a measure of tail current. Inward K^+ currents were studied using a ramp waveform which was applied over a range of potentials from –120 mV to -50 mV (at a rate of 1 mV/ms). The current response to the ramp could also be used as a measure of the reversal potentials of inward K^+ currents which were measured at the point of intersection of the generated current with the zero-current level. The low-pass filter was set at 1 or 2 kHz with data sampled at 5 kHz. All recordings were made at room temperature (21-24°C) and results are presented as mean ± SEM.

The bath solution contained (in mM): NaCl, 140; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1; glucose, 10; HEPEs, 10; pH adjusted to 7.3. The pipette solution contained (in mM): KCl, 140; NaCl,10; $CaCl_2$, 1; EGTA, 11; HEPES, 10; pH adjusted to 7.3.

4. Materials

Fura-2 was purchased from Molecular Probes (Eugene, OR). All other chemicals and materials were obtained from Sigma or Fisher Scientific (Vancouver, B.C.). ATP, ACh, carbachol and atropine were prepared by dissolving in HBSS. TNF- α was prepared in PBS as stock solution. DMSO was used to dissolve the Tg; after dilution the final concentration of DMSO in the 1 μM Tg solution was 0.1%.

Chapter Three RESULTS

Cultured human microglia had two general types of cell morphology. Fig. 2 shows phase contrast microscopy of these two cell types: a ramified form and an ameboid form. In this study, both types of cells were used for fluorescence imaging experiments and electrophysiological recordings. The responsiveness of individual microglia was similar in the whole population and was not correlated with a particular cell morphology.

1. Resting $[Ca^{2+}]_i$ level

The basal $[Ca^{2+}]_i$ level of human microglia was relatively stable. Their mean basal value of $[Ca^{2+}]_i$ was 59 ± 8 nM, n = 27 cells. We also measured the basal $[Ca^{2+}]_i$ when cultured microglia were incubated in Ca^{2+}-free HBSS buffer for over than 1 h. This was achieved by using Ca^{2+}-free HBSS for the whole process of fura-2/AM loading and its de-esterification as described above. The mean basal value of $[Ca^{2+}]_i$ after Ca^{2+}-free HBSS incubation was 13 ± 7 nM, n = 24 cells. The difference between the basal $[Ca^{2+}]_i$ level of microglia incubated in control HBSS and Ca^{2+}-free HBSS was statistically significant ($P<0.001$), and suggested that Ca^{2+}-free medium can induce Ca^{2+} depletion in human microglia. No further investigations were done on whether longer duration of incubation in Ca^{2+}-free media can induce more $[Ca^{2+}]_i$ decline.

2. Effects of high K^+ on $[Ca^{2+}]_i$ in human microglia

Elevated K^+ represents a putative stimulus for microglia. Changes in extracellular K^+ will change the membrane potential and may influence the $[Ca^{2+}]_i$, especially if voltage-dependent

Fig. 2 Two morphological forms of cultured human microglia. Vertical arrow in the left picture shows a ramified type of microglia; horizontal arrow in the right picture shows an ameobiod type of microglia. Phase contrast, 200X.

Ca^{2+} channels are present. Fluorescence imaging was carried out to examine the effects of high K^+ on $[Ca^{2+}]_i$ in microglia. After establishment of a stable baseline, a modified HBSS containing 50 mM K^+ (replacement of NaCl) was applied to the microglia. As shown in Fig. 3, high K^+ had no effect on $[Ca^{2+}]_i$. Similar results were obtained in another two experiments. The lack of effect of K^+ on $[Ca^{2+}]_i$ suggests that human microglia do not contain VDCCs. We did not investigate whether this protocol altered the Na^+/Ca^{2+} pump activity in these cells.

3. Effects of ATP on $[Ca^{2+}]_i$ in cultured human microglia

The $[Ca^{2+}]_i$ responses of cultured human microglia to ATP were investigated in this study. Only cells which had a stable baseline $[Ca^{2+}]_i$ value were studied. This was arbitrarily defined as a change of $[Ca^{2+}]_i$ less than 15 nM over 3 - 4 min. To exclude mechanical stimulation as a cause for changes in $[Ca^{2+}]_i$, the recording buffer was sometimes changed before ATP application. Occasionally, cells were incubated in serum-free media for 1-2 hours before exposure to agonists to evaluate the effect of serum on the $[Ca^{2+}]_i$ response. This was not evaluated statistically, however, no clear effect of serum was observed. ATP solutions in HBSS, with or without Ca^{2+}, were prepared immediately before use. The effects of ATP (100 μM) on microglia were studied in 10 separate experiments. Our data showed that 80 out of 88 microglia (91%) had evident changes in $[Ca^{2+}]_i$. A typical response shown in fig. 4 consisted of a transient $[Ca^{2+}]_i$ increase following ATP application. This result indicated functional purinoceptors exist in cultured human microglia. The most evident feature of the response shown was the rapid transient increase in $[Ca^{2+}]_i$ immediately following ATP application lasting for about 4 –5 min, where the peak level of $[Ca^{2+}]_i$ reached about 410 ± 50 nM, n=10. This indicates a rise of 351 ± 26 nM above the baseline $[Ca^{2+}]_i$ of 59 ± 8 nM. A more persistent plateau level of $[Ca^{2+}]_i$ following the peak transient was seen with a value of 110 ± 15 nM, n=10, higher than the resting

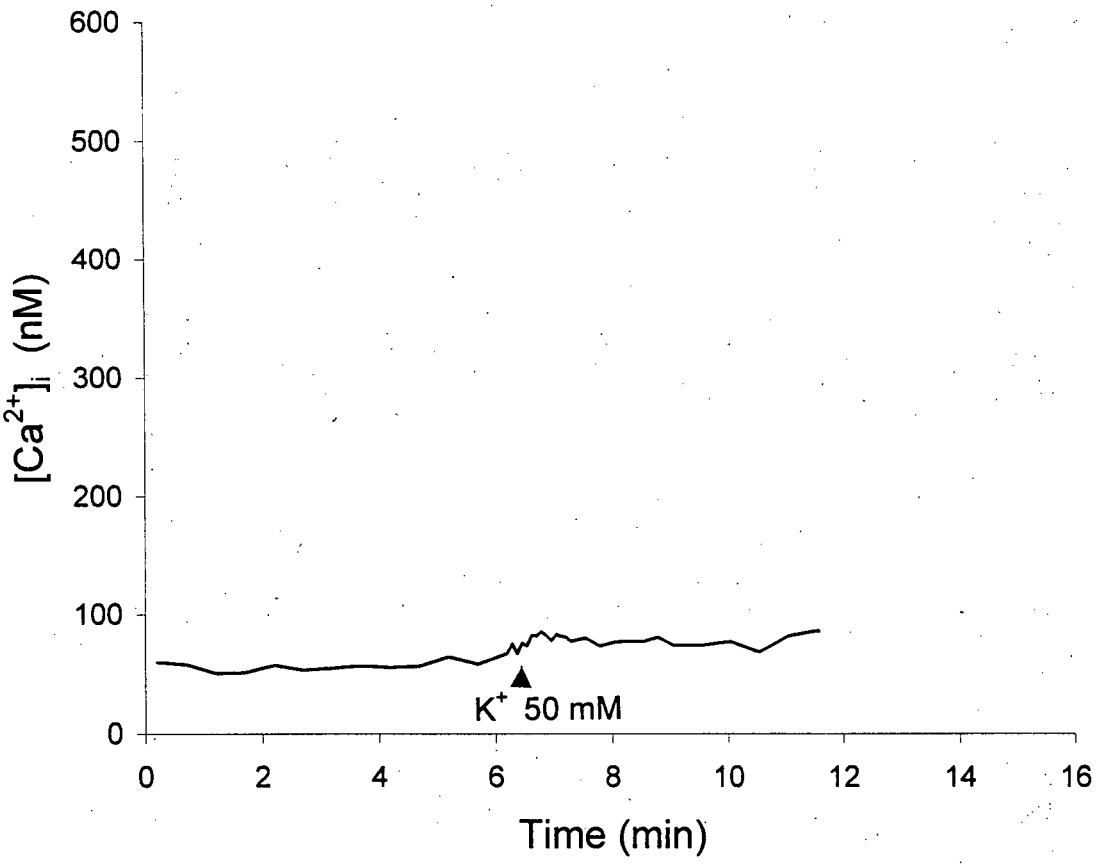


Fig 3 High K^+ (50 mM) has no effect on $[Ca^{2+}]_i$ in cultured human microglia. Microglia were recorded in 1.8 mM Ca^{2+}-containing HBSS. High K^+ was applied by using the same buffer but with K^+ 50 mM replacing Na^+. The trace showed no change after the K^+ application.

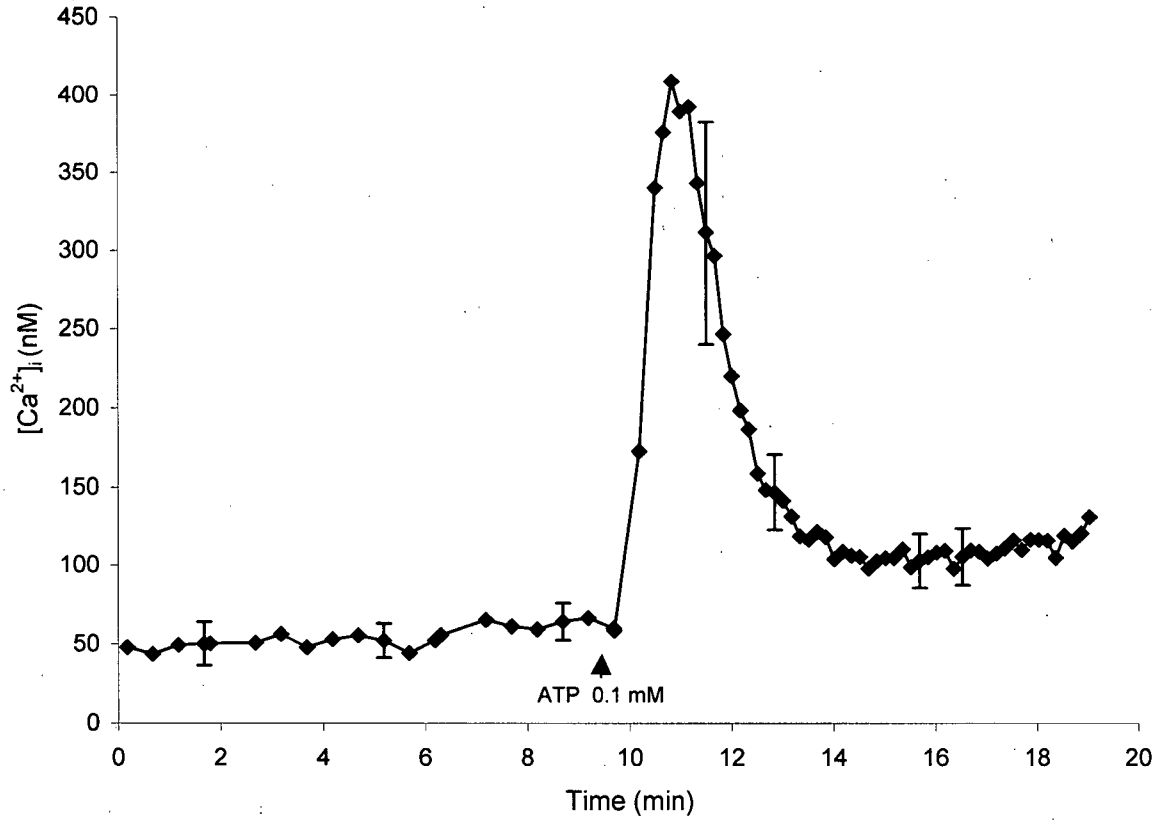


Fig 4 Effect of ATP (0.1 mM) on $[Ca^{2+}]_i$ of cultured human microglia. After a stable baseline (around 50 nM $[Ca^{2+}]_i$) was established, microglia were exposed to ATP which produced a rapid and transient increase in $[Ca^{2+}]_i$, to around 400 nM. This transient gradually recovered to a plateau level with a value (about 100 nM) higher than the resting level. Trace represents the average of 7 cells in a single experiment.

level ($P<0.001$). This plateau suggested the presence of an additional component in the ATP-induced change in $[Ca^{2+}]_i$.

3.1 $[Ca^{2+}]_i$ response of microglia to repeated exposure of ATP

Purinogenic responses are often characterized by receptor desensitization. This feature was investigated by two applications of ATP (100 μM) to the microglia where the Ca^{2+} concentration in HBSS was maintained at 1.8 mM. The two horizontal bars shown in Fig. 5 indicate the period of ATP application. This figure shows that the first ATP application produced a large transient $[Ca^{2+}]_i$ increase, reaching nearly 500 nM, and recovering to a higher value than baseline. A second ATP application induced a second $[Ca^{2+}]_i$ transient having a peak at about 200 nM. Thus, the second peak had a magnitude of only about 30% of the initial response peak. These two ATP applications were separated by about 9 min (Fig. 5). Similar effects were observed in 5 separate experiments separated by 9-10 min, the first ATP stimulation induced $[Ca^{2+}]_i$ transient peak is 540 ± 23, and the second one is 215 ± 12 ($P<0.001$). These findings suggested the possibility of purinoceptor desensitization in cultured human microglia.

3.2 The source of the ATP-induced $[Ca^{2+}]_i$ increase

To characterize the source of the ATP-induced $[Ca^{2+}]_i$ increase, microglia were recorded in Ca^{2+}-containing HBSS first, then, a rapid media change to Ca^{2+}-free HBSS was performed and ATP (100 μM) was applied to the recording bath. This less than 2 min Ca^{2+}-free HBSS was done to try to minimize any depletion of Ca^{2+} from intracellular Ca^{2+} stores, but permit removal of extracellular Ca^{2+}. The data showed that ATP stimulation in the absence of extracellular Ca^{2+} also induced a transient $[Ca^{2+}]_i$ increase. This response had a similar amplitude peak to the ATP response produced in Ca^{2+}- containing HBSS , but a more rapid recovery phase, around 2 min, compared to that obtained with external Ca^{2+} present (4-5 min). As shown in Fig. 6, the first

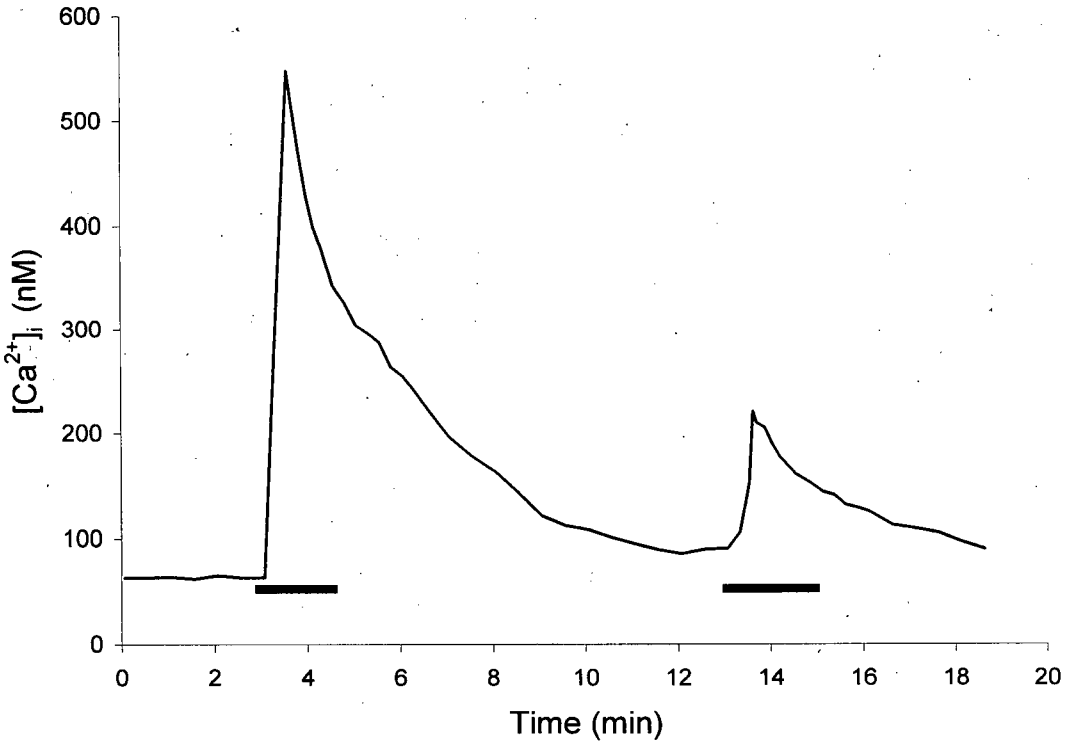


Fig. 5 Repeated application of ATP to cultured microglia in fura-2 fluorescence imaging. The horizontal bars indicate the duration of ATP (0.1 mM) applications. The interval between two applications is about 10 min. The second ATP stimulaiton produced a smaller $[Ca^{2+}]_i$ increase than the first application, and its amplitude reached only about 30% of the first peak.

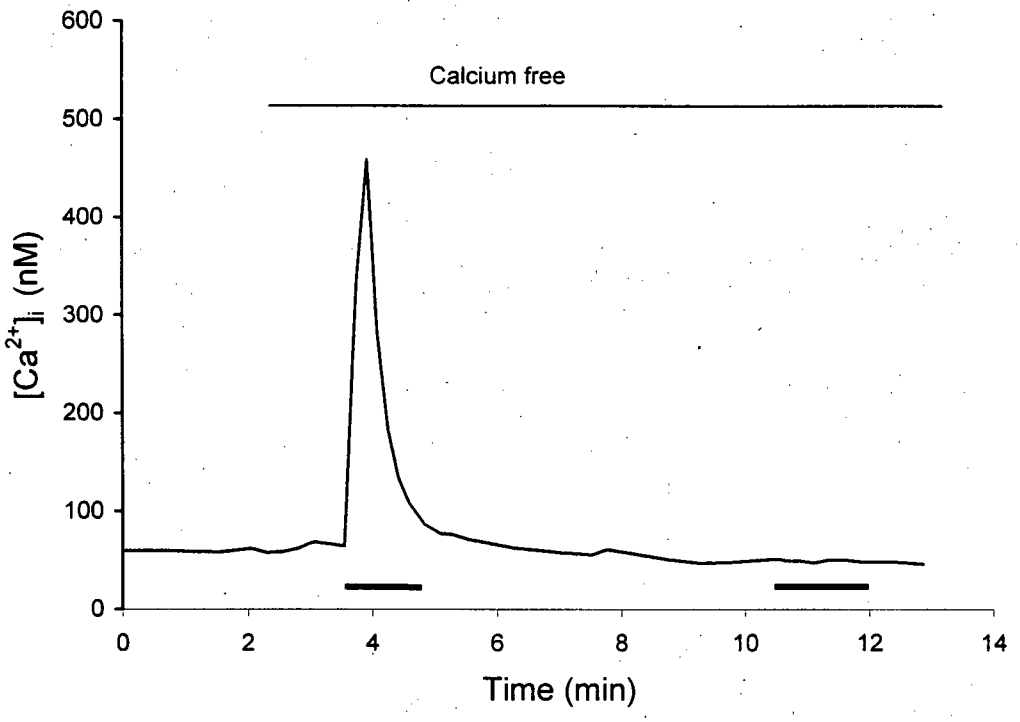


Fig. 6 ATP-induced release of Ca^{2+} from intracellular stores. After a stable baseline was established in Ca^{2+}-containing HBSS, the recording bath was changed to Ca^{2+}-free HBSS (upper horizontal bar). ATP applications were shown by lower horizontal bars. The first ATP (0.1 mM) application induced a rapid transient $[Ca^{2+}]_i$ increase which exceeded 400 nM and recovered to baseline level over around 1 min. The second ATP application failed to produce any $[Ca^{2+}]_i$ increase.

ATP stimulation in Ca^{2+}-free media produced a rapid $[Ca^{2+}]_i$ transient without an evident plateau phase, and the second ATP application (9 min after the first one) did not produce any response. Similar results were obtained in 5 different coverslips. The data suggest that the robust initial increase in $[Ca^{2+}]_i$ induced by the application of ATP was mediated by the release of Ca^{2+} from intracellular Ca^{2+} stores. In contrast, the small plateau component of the ATP response in Ca^{2+}-containing HBSS likely results from the entry of $[Ca^{2+}]_i$ across the plasma membrane.

4 Effects of ATP on membrane currents

4.1 Depolarizing ATP current:

Whole-cell patch-clamp recording was used to investigate the effects of ATP on microglial membrane currents. Application of 100 μM ATP to cultured human microglia led to activation of a transient inward depolarizing current. Fig. 7 shows a typical ATP-induced inward current. Measurement of inward currents were made from whole-cell recordings of 17 cells. The average peak amplitudes of these currents was 130 ± 21pA (range from 60 to 220 pA). In all cells recorded, the inward current induced by ATP was transient and returned to baseline within a few minutes, even in the continuing presence of ATP. The reversal potential for the transient current appeared to be near 0 mV since no ATP-induced inward current was evident if the holding potential was reduced to –20 mV.

4.2 Outward K^+ current

Under control conditions, the application of depolarizing steps from a holding potential of –60 mV did not generate evident outward currents (currents were generally less than 40 pA with steps to +40 mV) as shown in Fig. 8A. Following the application of ATP (100 μM), the amplitudes

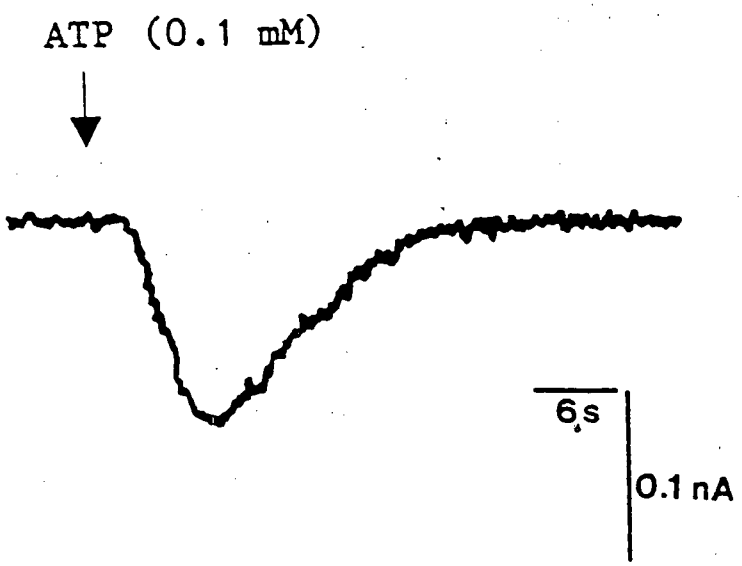


Fig. 7 Application of ATP (0.1 mM) to cultured human microglia induced a transient inward depolarizing current. ATP was applied at the time shown for the duration of the recording. The delay in response following ATP application is mainly due to time for perfusion solution to reach the cell.

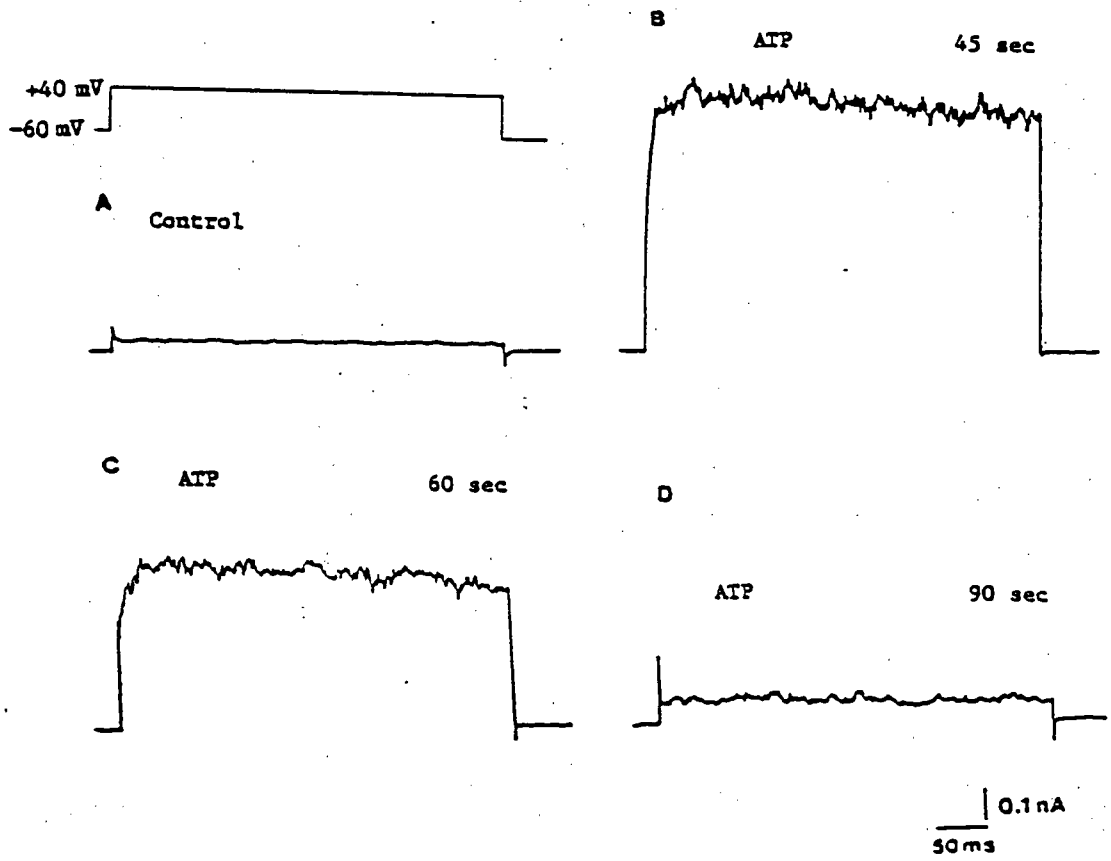


Fig. 8 Outward K^+ current (Ik) in human microglia with depolarization steps to +40 mV. (A) Ik in control solution. (B) Effects of ATP (0.1 mM) on Ik after 45 sec of ATP perfusion. (C) Ik after 60 sec of ATP perfusion. (D) Ik after 90 sec of ATP perfusion. In all recordings the holding potential was –60 mV.

of the outward currents were significantly enhanced, where the current amplitude was near 1 nA at 45 sec (Fig. 8B). In the continuing presence of ATP, the outward current then gradually decreased with time, and the peak amplitude recorded 60 sec after the application of ATP was about half of that recorded 45 sec following ATP exposure (Fig. 8C). The current diminished further with time and after 90 sec the outward current was near control level (Fig. 8D). Similar ATP-induced outward K^+ currents were recorded from 12 cells and the mean current amplitude was 1.16 ± 0.04 nA (measured at 45 sec after ATP application) compared with control currents of 36 ± 7 pA. Thus, ATP perfusion enhanced outward K^+ current by about 30-fold. A decline in the ATP-induced outward K^+ current with time was consistently observed in all cells, however, the time course of decrease was highly variable between cells.

The effects of ATP (100 μM) on the reversal potential of this outward current were tested by using an applied voltage ramp between –110 mV and 0 mV (Fig. 9). A small outward current was observed under control conditions which had a reversal potential near –10 mV (indicated by arrow in Fig. 9A). After 60 sec of ATP application, an outward current developed with a reversal potential estimated at –78 mV when the same voltage ramp was applied (Fig. 9B). This reversal potential was close to –80 mV and thus was likely selective for outward movement of K^+.

4.3 Inward K^+ current

In order to study the effects of ATP on inward K^+ current, hyperpolarizing steps or ramps from a holding potential level of –60 mV were applied. This ramp protocol can vary the potential over the range from –120 mV to –50 mV at a rate of 1 mV/ms, which allowed for a rapid estimation of the conductance and the reversal potential of inward K^+ current. This ramp protocol was applied both in control microglia and in 100 μM ATP -treated microglia. Representative results from one

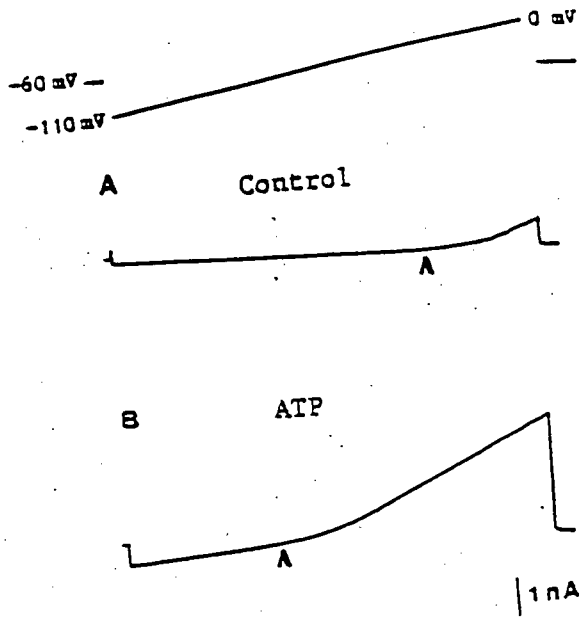


Fig. 9 Effects of ATP (0.1 mM) on the Ik reversal potential. (A) A ramp waveform was applied as shown (range from –110 mV to 0 mV, 1 mV/ms) and Ik was recorded under control conditions; the arrow indicates reversal potential, -10 mV. (B) In the presence of ATP, an outward Ik was enhanced and the reversal potential was shifted to the left (-75 mV) relative to control.

experiment are shown in Fig. 10, in control solution, this channel had a conductance of 1.4 nS and an estimated reversal potential of –92 mV. This value was close to the expected reversal potential for K^+ with the solutions employed in this experiment. In the presence of ATP, the slope conductance was increased to 2.6 nS and the reversal potential was shifted to –72 mV. Thus, in this case ATP depolarized the cell by 20 mV. Similar experiments on the effects of ATP on properties of inward K^+ current were performed and analysed in 10 cells. The results showed ATP increased the slope conductance by a factor of 2.4 (slope conductance in ATP and control of 3.4 ± 0.3 nS and 1.0 ± 0.3 nS, respectively); and shifted the reversal potential value in the depolarizing direction by a mean value of 17 ± 5 mV, from –85 ± 6 mV in control to –68 ± 4 mV with ATP. No statistical analysis was performed, but the conductance increase and reversal potential shift were likely due to the effects of ATP.

5. Effects of cholinergic agonists on $[Ca^{2+}]_i$ in cultured human microglia.

5.1 Acetylcholine and CCh induce a transient $[Ca^{2+}]_i$ increase in human microglia

We studied the $[Ca^{2+}]_i$ responses of microglia to CCh in a total of 77 cells and to ACh in a total of 27 cells. A CCh concentration of 100 μM was chosen to achieve a maximal CCh response. CCh application to microglia in 1.8 mM Ca^{2+}-containing HBSS produced a rapid, transient increase in $[Ca^{2+}]_i$. Figure 11 showed the CCh-induced $[Ca^{2+}]_i$ response had a rise time of less than 1 min and recovered within 4 min. This trace appeared to be composed of two phases: an initial $[Ca^{2+}]_i$ spike followed by a gradually recovering plateau phase. The CCh-induced increases in $[Ca^{2+}]_i$ were observed in 44 out of 77 (58.5%) cultured microglia. Co-application of CCh and atropine (10 μM), an inhibitor of muscarinic receptors, did not produce any $[Ca^{2+}]_i$ response (Fig. 12) in CCh-

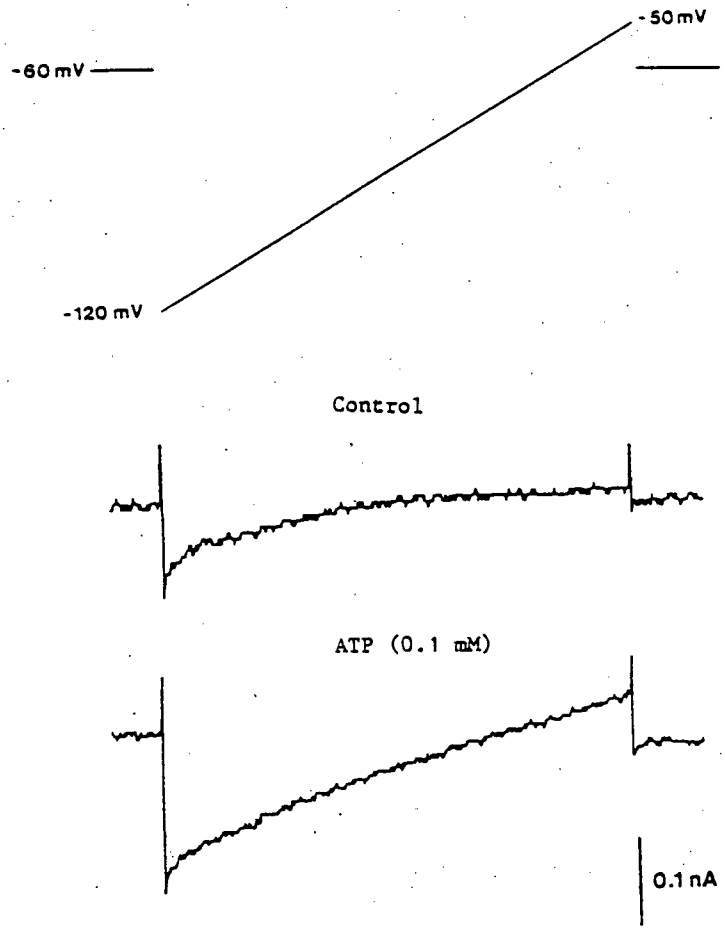


Fig. 10 Human microglia showed an inward K^+ current, and its conductance can be increased by application of ATP (0.1 mM). The holding potential of the whole-cell recording was –60 mV. Applied ramp protocol was in the range of voltage change from –120 mV to –50 mV. In control microglia, an inward K^+ current was observed; after application of ATP to the cell, the slope conductance of this inward current increased significantly.

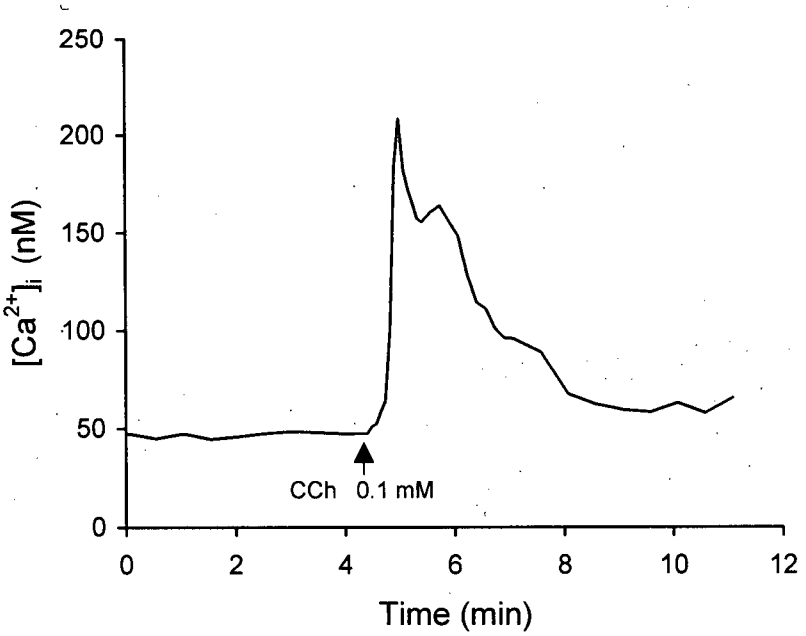


Fig. 11 Effects of CCh (0.1 mM) on $[Ca^{2+}]_i$ in cultured human microglia. Application of CCh induced a transient increase in $[Ca^{2+}]_i$, which reached a peak of 210 nM and then gradually recovered near to basal levels within 4 min. The recording was done in 1.8 mM Ca^{2+}-containing HBSS.

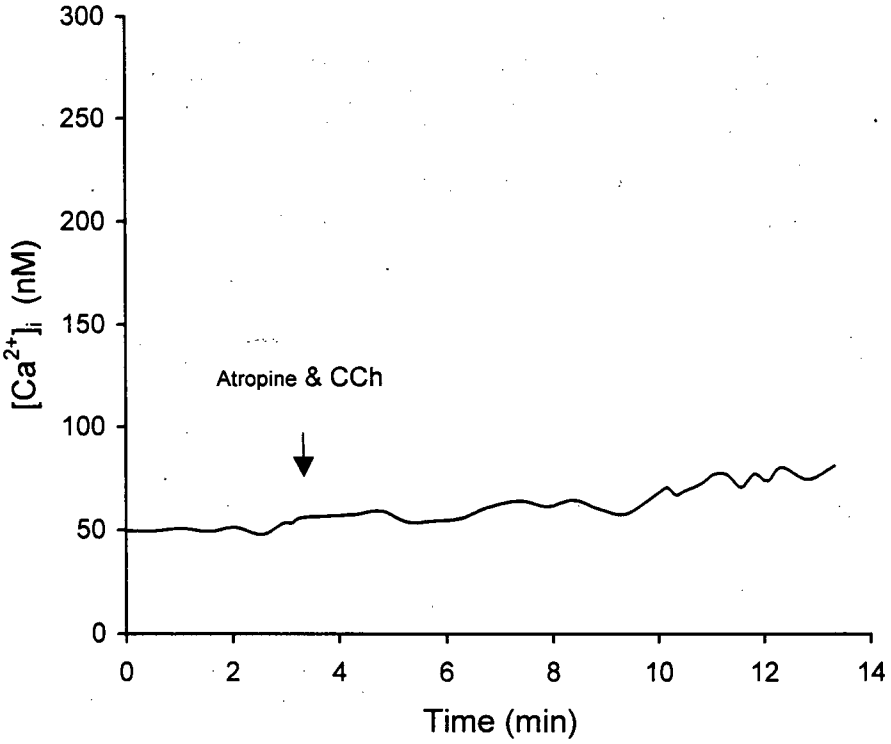


Fig. 12 Co-application of CCh (0.1 mM) and atropine (0.01 mM) did not produce any $[Ca^{2+}]_i$ changes in cultured microglia.

responsive cells indicating that CCh works through muscarinic receptors (n = 42 cells). Acetylcholine (100 μM) also produced a transient response in $[Ca^{2+}]_i$ as shown in fig. 13. Overall, ACh was applied to 27 microglia and 17 of these cells (63%) had responses similar to that shown in Fig. 13. In subsequent studies we used CCh to investigate further the properties of this $[Ca^{2+}]_i$ signal.

5.2 Carbachol-induced $[Ca^{2+}]_i$ response desensitization.

In order to determine if cholinergic stimulation exhibited any desensitization, successive applications of CCh (100 μM) were performed on CCh-responsive microglia. A second application of CCh given 5-6 minutes after the first application produced a response with an amplitude of approximately 50 - 60% of the initial $[Ca^{2+}]_i$ peak. Figure 14 shows a representative example of the $[Ca^{2+}]_i$ response to repeated CCh applications in cultured microglia, which were observed in total of 5 experiments with average first peak 235.3 ± 10.24, and the second one 156.75 ± 7.76 ($P<0.001$). This suggests that microglia desensitize to repeated exposure of CCh.

5.3 The source of CCh-induced $[Ca^{2+}]_i$ increase in human microglia

To determine the source of the CCh-induced $[Ca^{2+}]_i$ increase in human microglia, we examined the influence of extracellular Ca^{2+}. After obtaining a stable baseline in 1.8 mM Ca^{2+}-containing HBSS, microglia were exposed to Ca^{2+}-free HBSS for a short period (less than 2 min), then CCh (100 μM) was applied. This CCh application produced a transient $[Ca^{2+}]_i$ response with peak response value of 240.16 ± 26.36 which had no significant difference ($P>0.05$) with that recorded in the presence of external Ca^{2+}. These analysis were based on the results of 21 cells in 3 independent experiments. This transient recovered over 2 min and recovered to a lower value than the basal level. The second CCh application 5–6 min later in gave no response (Fig. 15). These

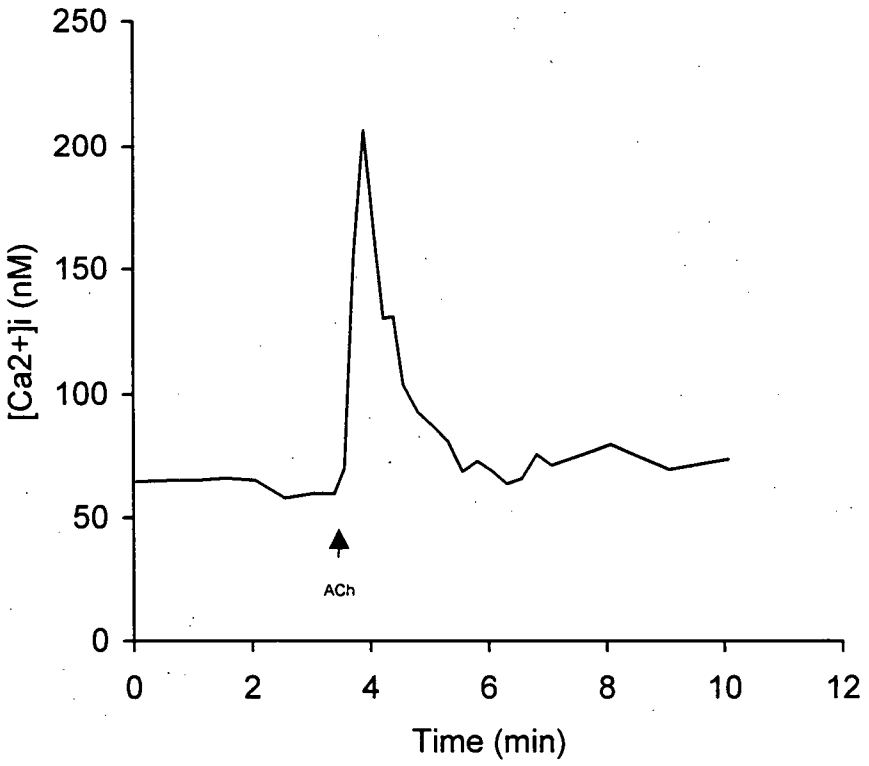


Fig. 13 ACh (0.1 mM) stimulated a transient [Ca2+]i increase in cultured human microglia. Experiment performed in 1.8 mM Ca2+ containing HBSS recording bath, and arrow indicated the time ACh was applied. [Ca2+]i reached 206 nM immediately after ACh application, and gradually returned near to baseline levels within 2 min.

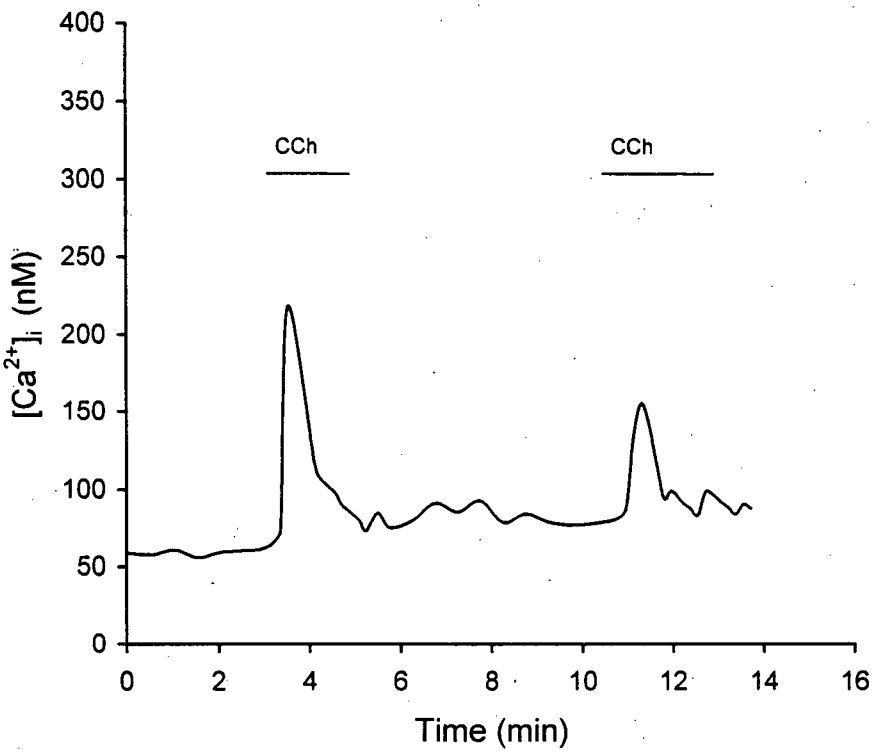


Fig. 14 Successive applications of CCh to microglia induced a decrease in the $[Ca^{2+}]_i$ response. Experiments were performed in 1.8 mM Ca^{2+}-containing HBSS. CCh (0.1 mM) stimulation induced a transient increase in $[Ca^{2+}]_i$, which reached approximately 220 nM . A second application of CCh at the same concentration applied 5-6 min later produced a transient that reached an amplitude of only 50-60% of the amplitude of the first transient.

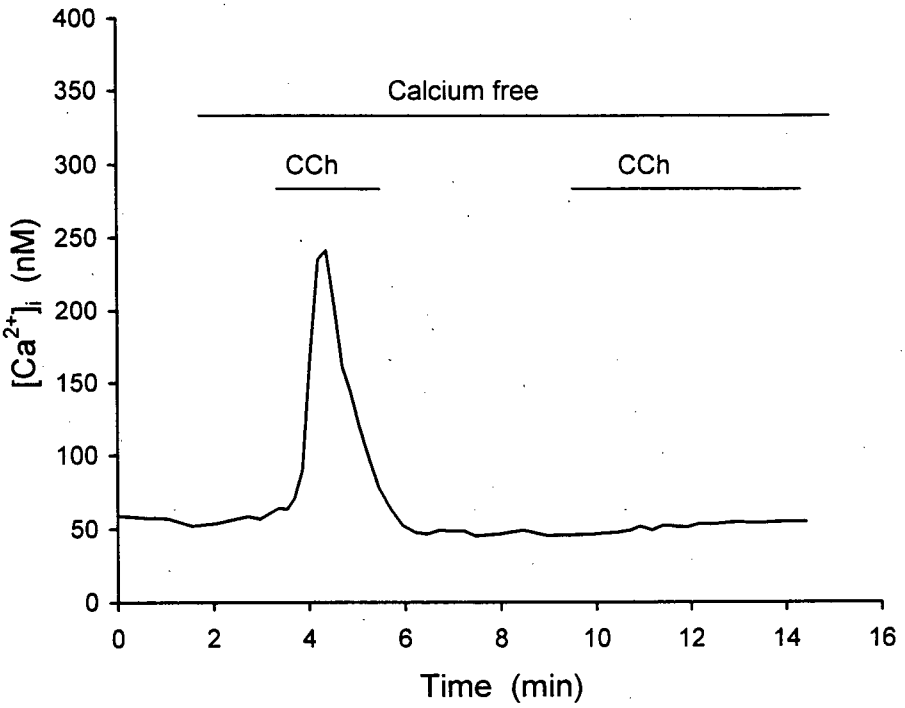


Fig. 15 Carbachol-induced $[Ca^{2+}]_i$ increase is from the release of Ca^{2+} from intracellular stores. After obtaining a stable baseline in 1.8 mM Ca^{2+}-containing HBSS the recording bath was changed to Ca^{2+}-free HBSS, and CCh (0.1 mM) was applied to microglia within 2 min. Carbachol stimulated a transient increase of $[Ca^{2+}]_i$ which was similar to the one observed in Ca^{2+}-containing HBSS. A second CCh application did not induce any response.

results suggest that the change in $[Ca^{2+}]_i$ resulting from CCh stimulation is largely mediated by release from intracellular Ca^{2+} stores as the response was observed in Ca^{2+}-free HBSS, and a second CCh application produced no response. As with the ATP-induced $[Ca^{2+}]_i$ response, the second CCh-induced $[Ca^{2+}]_i$ increase was dependent on the presence of extracellular Ca^{2+} and the absence of a $[Ca^{2+}]_i$ response upon the second CCh application might be due to of the failure of refilling of the emptied intracellular Ca^{2+} stores.

6. Effects of extracellular Ca^{2+} on $[Ca^{2+}]_i$ in human microglia

6.1 Effects of Ca^{2+} store release or depletion on $[Ca^{2+}]_i$ in human microglia

As mentioned above, the basal $[Ca^{2+}]_i$ of microglia was different when in Ca^{2+}-containing and Ca^{2+}-free HBSS. To explore the role of $[Ca^{2+}]_o$ on the basal $[Ca^{2+}]_i$ levels of microglia, a modified fura-2 loading procedure was used as follows: beginning with the fura-2 dye loading procedure, Ca^{2+}-free HBSS was used for the loading buffer and microglia were then washed and de-esterified in the Ca^{2+}-free HBSS. Microglia were studied after more than 1 h in Ca^{2+}-free incubation. At this point, microglia had a resting $[Ca^{2+}]_i$ level of 13 – 18 nM (Fig. 16), and did not produce any changes in $[Ca^{2+}]_i$ when they were exposed to ATP or CCh (n = 5). This might occur because the internal Ca^{2+} stores had been depleted after incubation in Ca^{2+}-free media for 1 h. In this case, when 1.8 mM Ca^{2+}-containing HBSS was restored to the recording bath, rapid and high amplitude $[Ca^{2+}]_i$ transients were elicited, however they showed different amplitudes and time course among individual cells as shown in figure 16. Similar results were observed in 32 cells in 4 separate experiments. These results suggested that restoration of extracellular Ca^{2+} induced a large Ca^{2+} influx, which might be through the mechanism of capacitative Ca^{2+} entry. In order to avoid intracellular Ca^{2+} store depletion when cells were studied in Ca^{2+}-free HBSS,

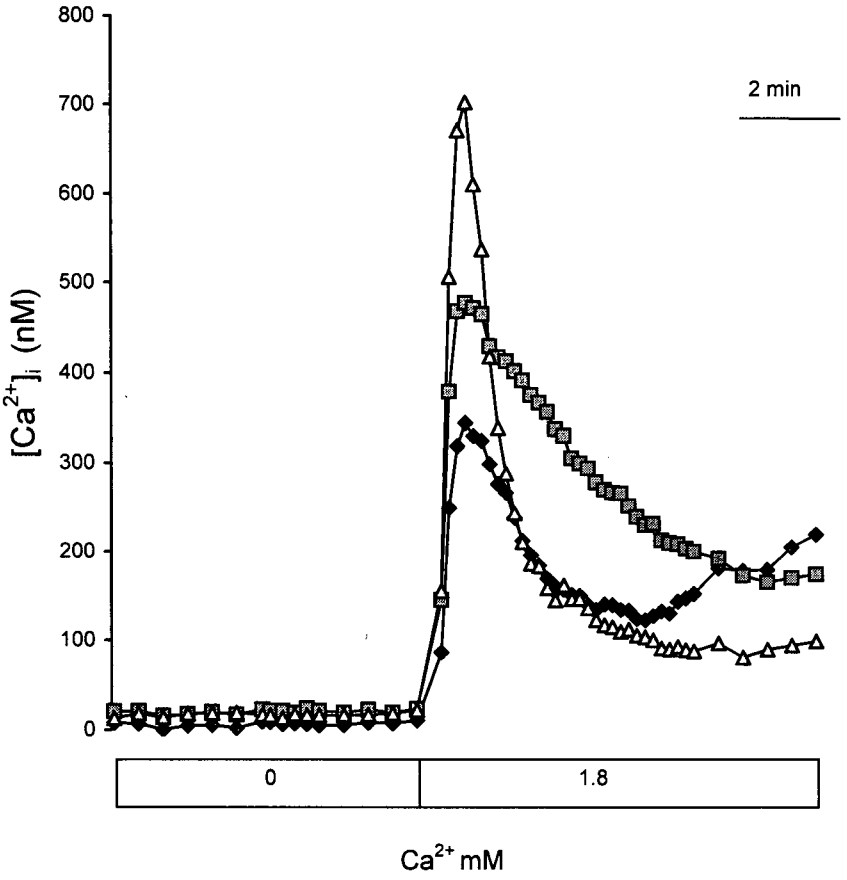


Fig. 16 Levels of $[Ca^{2+}]_i$ in 3 individual cells. Microglia were incubated in Ca^{2+}-free HBSS for more than 60 min. Under these conditions, $[Ca^{2+}]_i$ was 13 - 18 nM. Replacement with 1.8 mM Ca^{2+}-containing HBSS (as shown at bottom) produced a large $[Ca^{2+}]_i$ increase having different amplitudes in microglia.

the coverslips with microglia were washed and exposed to Ca^{2+}-free HBSS for less than 2 min duration, then ATP or CCh containing HBSS was applied to the cells. A robust increase in $[Ca^{2+}]_i$ was always observed when 1.8 mM Ca^{2+}-containing HBSS was restored to the bath following either ATP (100 μM) (Fig. 17, 32 cells in 5 experiments), or CCh application (100 μM) (Fig. 18, 19 cells in 3 experiments). In these studies I found that whether or not microglia had $[Ca^{2+}]_i$ responses to ATP or CCh, large increase in $[Ca^{2+}]_i$ were always seen when Ca^{2+}-containing (1.8 mM) HBSS was used to replace Ca^{2+}-free HBSS. However, the magnitude of the $[Ca^{2+}]_i$ change was highly variable among cells. To further clarify the effects of extracellular Ca^{2+} on changes in $[Ca^{2+}]_i$, three experiments were performed by replacing Ca^{2+}-containing HBSS with Ca^{2+}-free HBSS for a brief period (3 – 4 min). All the cells demonstrated significant $[Ca^{2+}]_i$ increases when Ca^{2+}-containing HBSS was restored. The increase in $[Ca^{2+}]_i$ was different among individual cells (Fig. 19).

These data raised the possibility, that the extracellular Ca^{2+} change from 0 mM to 1.8 mM might be a signal to induce a $[Ca^{2+}]_i$ increase through the function of the calcium receptor (CaR). To evaluate this possibility, neomycin (50 μM) was applied to the recording bath after the microglia were exposed to Ca^{2+}-free HBSS for 3-5 min during the measurement, however, there was no evident response in 4 separate recording. No further investigation was performed by applying Gd^{2+} or measuring the mRNA of CaR in present study.

6.2 Effects of high $[Ca^{2+}]_o$ on the $[Ca^{2+}]_i$ in cultured human microglia

In order to further investigate if $[Ca^{2+}]_o$ changes affect $[Ca^{2+}]_i$ of microglia, 3.6 mM Ca^{2+}-containing HBSS was applied to cultured microglia following incubation in control (1.8 mM

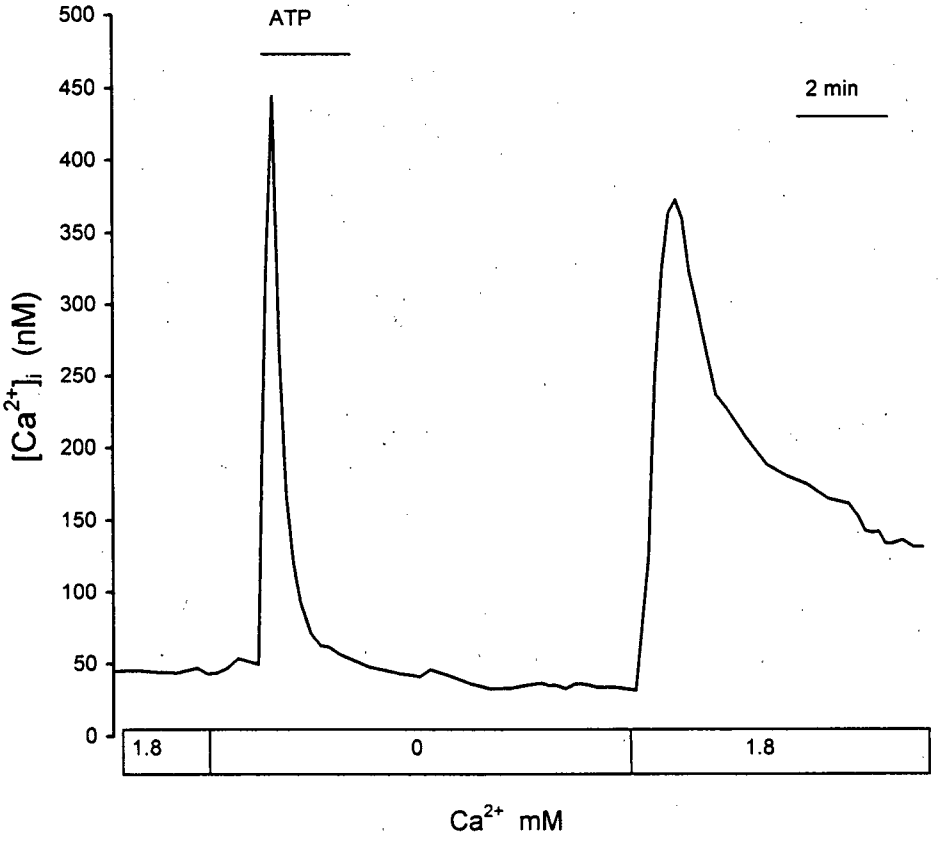


Fig. 17 Application of ATP (0.1 mM) (indicated by the top horizontal bar) to microglia in Ca^{2+}-free HBSS induced a transient $[Ca^{2+}]_i$ increase. Replacement of the buffer with 1.8 mM Ca^{2+}-containing HBSS elecited a robust $[Ca^{2+}]_i$ increase. The trace shown is representative of 32 cells in 5 experiments.

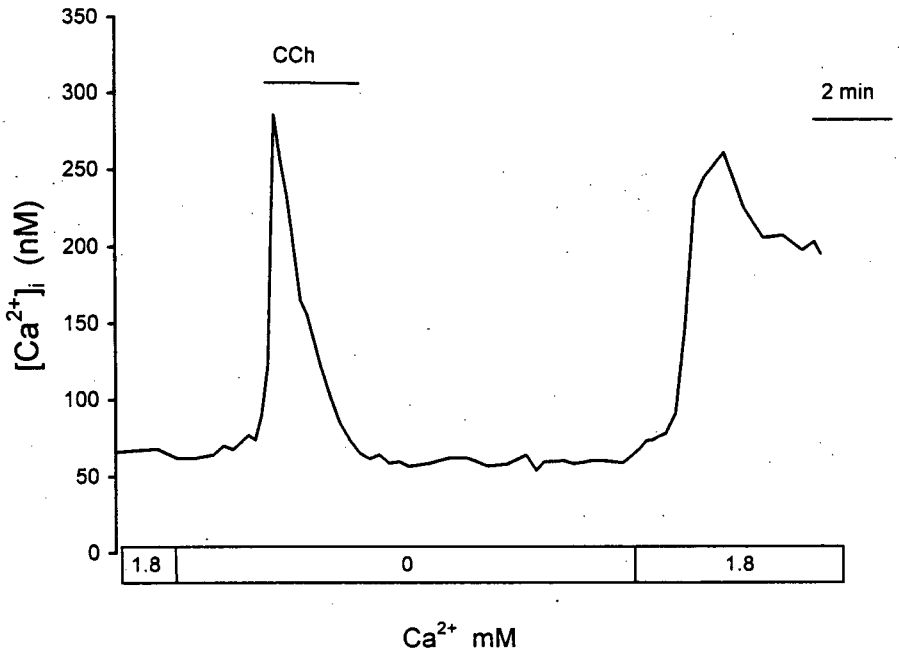


Fig. 18 Application of CCh (0.1 mM) to microglia in Ca^{2+}-free HBSS induced a transient $[Ca^{2+}]_i$ increase. Replacement of the recording buffer with 1.8 mM Ca^{2+}-containing HBSS produced a rise in $[Ca^{2+}]_i$. This trace is a representative of 19 cells in 3 separate experiments.

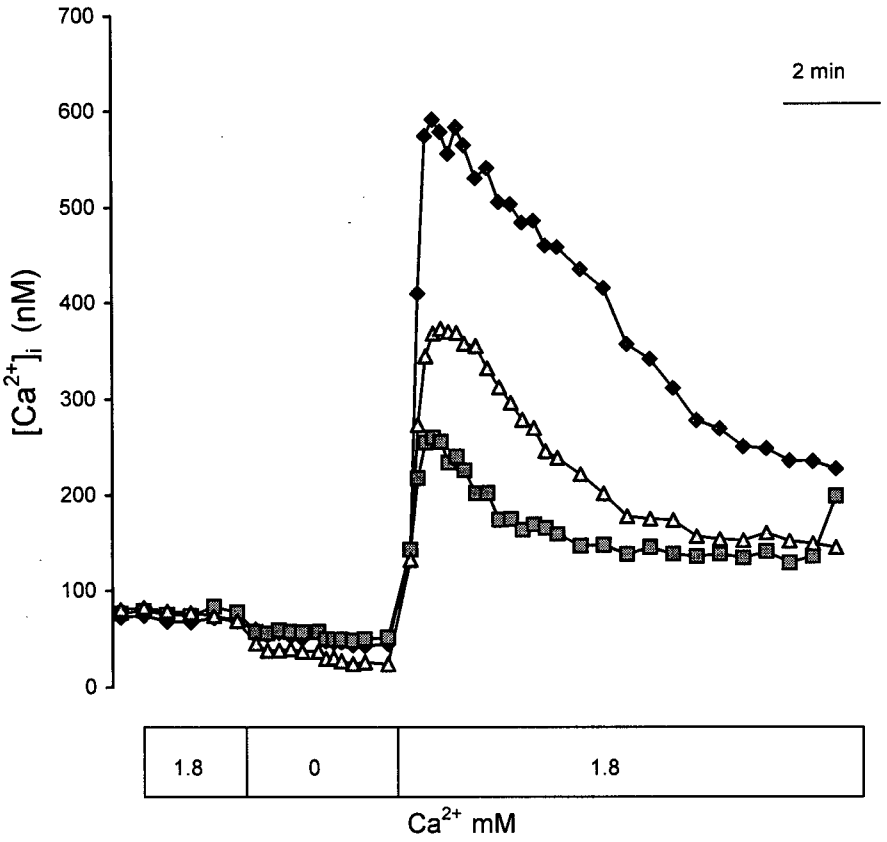


Fig. 19 Incubating the cultured microglia for 3.5 min in Ca^{2+}-free HBSS induced large $[Ca^{2+}]_i$ increase when external Ca^{2+} was replaced.

Ca^{2+}-containing HBSS). The results of this experiment showed that $[Ca^{2+}]_i$ gradually increased when $[Ca^{2+}]_o$ was changed from 1.8 mM to 3.6 mM, and this increase was reproducible in the same cell (data not shown). We did not perform further investigations of this effects.

6.3 Effects of thapsigargin on $[Ca^{2+}]_i$ of human microglia

Thapsigargin is a blocker of the Ca^{2+} pump on ER, and it release Ca^{2+} from internal stores, thus depleting the stores. Application of Tg (1 μM) to microglia in 1.8 mM Ca^{2+}-containing HBSS initially led to an increase in $[Ca^{2+}]_i$, followed by a progressive increase in $[Ca^{2+}]_i$ (Fig. 20A). In another experiment, Tg (1 μM) was applied to microglia in Ca^{2+}-free HBSS. Same amount release of $[Ca^{2+}]_i$ was observed which returned to the resting level (Fig. 20B); at this point, 1.8 mM Ca^{2+}-containing HBSS was re-introduced, and this triggered a robust $[Ca^{2+}]_i$ increase (Fig. 19B). Based on these results, it is likely that the initial transient increase in $[Ca^{2+}]_i$ induced by Tg resulted from release of Ca^{2+} from intracellular Ca^{2+} stores (store depletion); whereas the high plateau level of $[Ca^{2+}]_i$ may have occurred from Ca^{2+} influx, as the $[Ca^{2+}]_i$ plateau was not present when the cells were studied in Ca^{2+}-free HBSS.

Chapter Four DISCUSSION

1. Resting $[Ca^{2+}]_i$ level of microglia

Cultured human microglia had a resting $[Ca^{2+}]_i$ level of 59 ± 8 nM (n=27), which is similar to that observed in rat microglia with the value of 68.1 ± 2.8 nM in one report (Korotzer et al., 1995) and around 50 nM in another study (Whittemore et al., 1993). The study of Korotzer et al (1995) also implied that microglia with high $[Ca^{2+}]_i$ baseline levels (>100 nM) are unstable under control conditions.

The mean basal $[Ca^{2+}]_i$ level of microglia in Ca^{2+}-free media was lower than that in Ca^{2+}-containing media. Similar results have been observed in microglial cell lines (Righi et al., 1995). This indicates that $[Ca^{2+}]_i$ was dependent in part on extracellular Ca^{2+}. A similar dependence of $[Ca^{2+}]_i$ on extracellular Ca^{2+} has been observed previously in mammalian lymphocytes, where the addition of Ca^{2+} to cells in Ca^{2+}-free solutions resulted in a rise in $[Ca^{2+}]_i$ to a steady state value, and the return of cells to a Ca^{2+}-free solution produced a fall in $[Ca^{2+}]_i$ (Tsien et al., 1982). The dependence of $[Ca^{2+}]_i$ on $[Ca^{2+}]_o$ has also been reported for keratinocytes (Kruszewski et al., 1991), rat hippocampal neurons (Tymianski et al., 1993), the motor neuron cell line NSC-19 (Hasham et al., 1994) and cultured rat cortical neurons (Villalba et al., 1994). The variation of $[Ca^{2+}]_i$ with $[Ca^{2+}]_o$ has been suggested as being due to the operation of transmembrane Ca^{2+} flux, either by channel or transporters. In the motor neuron cell line NSC-19 the blockade of voltage-dependent Ca^{2+} channels with application of known antagonists did not alter the changes $[Ca^{2+}]_i$ with $[Ca^{2+}]_o$ (Hasham et al., 1994).

2. Voltage-gated Ca^{2+} channels in microglia

High K^+ application to human microglia produced no effect on $[Ca^{2+}]_i$. This was consistent with previous work showing that when mouse microglia were depolarized by increasing the bath K^+ concentration from the resting level of 5.4 mM to 55 mM, there was no apparent change in the level of $[Ca^{2+}]_i$, indicating a lack of voltage-activated Ca^{2+} channels in mouse microglia (Walz et al., 1993).

3. ATP modulates $[Ca^{2+}]_i$ and membrane currents in human microglia.

3.1 ATP produces a transient $[Ca^{2+}]_i$ increase in human microglia

The purinergic agonist, ATP, is a physiological regulator of monocyte $[Ca^{2+}]_i$ signaling; it can mobilize Ca^{2+} from intracellular stores (Cowen et al., 1989). In the present study, the ATP-stimulated $[Ca^{2+}]_i$ response in cultured microglia is a transient $[Ca^{2+}]_i$ increase which seemed to be composed of two phases: an initial $[Ca^{2+}]_i$ increase immediately following the ATP application and a delayed plateau with higher $[Ca^{2+}]_i$ value than the resting $[Ca^{2+}]_i$ level. Repeated applications of ATP to microglia induced a similar transient $[Ca^{2+}]_i$ increase, but with less response amplitude. This is likely an indication that the ATP response is desensitizing, as has been observed in electrophysiological studies in mouse microglia (Walz et al., 1993).

When experiments were performed in Ca^{2+}-free media, ATP application produced only an initial $[Ca^{2+}]_i$ spike, and no delayed plateau phase was observed. In addition, a second ATP application to the same culture failed to produce any $[Ca^{2+}]_i$ transient. Other studies on mouse microglia (Ferrai et al., 1996) and in a human leukemia cell line (Gardner et al., 1997) have also reported an ATP-induced $[Ca^{2+}]_i$ increase in a Ca^{2+}-free recording bath. This suggests that the initial $[Ca^{2+}]_i$ increase induced by ATP application is mediated by intracellular Ca^{2+} store release, as it occurs in the absence of extracellular Ca^{2+}. The plateau phase of the $[Ca^{2+}]_i$

response which occurred in Ca^{2+}-containing media is due to Ca^{2+} influx, as the ATP-induced $[Ca^{2+}]_i$ transient did not occur in Ca^{2+}-free media. Successive ATP responses are dependent on the presence of extracellular Ca^{2+}, and the failure to observe these responses in Ca^{2+}-free media might be due to the failure of replenishment of intracellular stores in the absence of extracellular Ca^{2+}. One previous study in rat brain microglia also showed that LPS can induce two successive $[Ca^{2+}]_i$ increases in Ca^{2+}-containing media, but only the first one in Ca^{2+}-free media (Bader et al., 1994).

A previous report on ATP effects on $[Ca^{2+}]_i$ in a microglial cell line postulated that most of the Ca^{2+} increase was generated by influx across the plasma membrane since removal of external Ca^{2+} greatly reduced the initial $[Ca^{2+}]_i$ spike (Ferrai et al., 1996). These results are different from what we observed in human microglia where the initial $[Ca^{2+}]_i$ spike values were almost the same, with or without extracellular Ca^{2+} present. Possible reasons for this difference could be species variation, or the sensitivity of InsP_3-induced Ca^{2+} release. Since available evidence suggests that InsP_3 sensitivity increases as the intracellular stores fill up with calcium (Berridge, 1993); the Ca^{2+} content of ER in cultured microglia could be affected in different culture conditions. Alternatively, it has been postulated that variations in InsP_3 sensitivity among different cells may depend upon InsP_3R receptor heterogeneity (Berridge, 1993).

P_2y receptors are widespread among cells, and may function in the control of $[Ca^{2+}]_i$ levels through intracellular Ca^{2+} store release. The present results suggest that ATP-induced $[Ca^{2+}]_i$ responses in cultured human microglia involves metabotropic purinoceptor (P_2y) activation, thus turning on the PLC-InsP_3 signaling pathway and triggering Ca^{2+} release from intracellular stores. This ATP-induced Ca^{2+} release was followed by Ca^{2+} influx, which has been

shown previously in mouse microglia and suggests mediation by ionotropic P_2x receptors (Ferrari et al, 1996).

3.2 ATP-induced electrophysiological properties of human microglia

In the present study, we showed that ATP application to microglia induced depolarizing inward cationic currents with a current reversal potential near 0 mV, suggesting the involvement of a nonselective cation conductance. The study of Ferrari et al (1996) using the microglial cell line N9, N13 and freshly isolated mouse microglia suggested that P_2z/P_2x_7 might exist in microglia, since microglia underwent an uptake of extracellular markers such as ethidium bromide and lucifer yellow upon stimulation with high concentrations of ATP. Electrophysiological studies have also been done in rat and mouse microglia and suggest that extracellular ATP activates a cation channel subtype purinoceptor, that in turn opens large nonselective cationic pores and potassium channels (Walz et al., 1993; Kettenmann et al., 1993; Norenberg et al., 1994a). Similar observations have also been reported in mouse brain slices following ATP treatment in situ (Haas et al., 1996). The finding of P_2z receptor proteins in N9 and N13 cells using Western blotting (Langosch et al., 1994) further confirmed that P_2z/P_2x_7 might exist in microglia. The characterization of P_{2Z}/P_2x_7 purinoceptors in microglia is still at a very early stage, and has not yet allowed identification of a physiological role, but it was reported to be modulated during cell cycles and coupled to IL-1 beta release (Ferrari et al., 1996). It has been shown previously that ATP elicits the activation of a cation conductance in vascular and smooth muscle cells (Nakazawa and Matsuki, 1987), skeletal muscle (Kolb and Wakelam, 1983), neurons (Krishtal et al.,1988), and macrophages (Steinberg & Silverstein, 1987).

The electrophysiological properties of microglia are dominated by inwardly rectifying K^+ channels and by the lack of outward currents (Kettenmann et al., 1993). The lack of outward currents in microglia has the functional consequence that a small inward current will lead to a large membrane depolarization, which could be an important signaling event in microglia. ATP-induced long lasting depolarization will therefore trigger subsequent cellular events. Our whole-cell patch-clamp data also showed that cultured human microglia expressed very small outward K^+ currents under control conditions. Application of ATP (0.1 mM) to the microglia can enhance this outwardly rectifying conductance significantly in 1 min with a reversal potential close to the K^+ equilibrium potential, indicating the activation of outward K^+ channels. Quick activation of the outward current implies that these channels may not be newly expressed, but are constructively present in the microglial membrane. Thus we assume that this outward K^+ channel is usually closed under resting conditions and can be activated by signals such as ATP receptor activation. It has been reported that the transition of microglia from a resting state to an activated state due to the application of LPS is associated with the expression of a outwardly rectifying K^+ channel (Ilschner et al., 1995). The outward conductance is activated rapidly and decreases considerably over 1.5 min following stimulation of purinoceptors. The present study also confirmed the presence of inward K^+ current in cultured human microglia; ATP stimulation can increase the conductance of this inward current. We therefore postulated that the activity of K^+ channels might be involved in the transition of microglia from a resting to an activate state, and this modulation could be controlled by ATP. K^+ channels can modulate basic cellular properties, such as membrane potential, which in turn influences a variety of cellular parameters like intracellular pH, $[Ca^{2+}]_i$ and $[Na^+]_i$.

3.3 Significance of ATP effects on microglia

Microglia are found throughout the brain where they play a major role as immunocompetent cells. Neurons are the cell types that have been shown to release ATP (Zimmerman, 1994). Large amounts of extracellular ATP signal pathological events like ischemia and injury, and this transmitter may be the molecular link between injured neurons and microglia. The present study showed that extracellular ATP can induce a transient increase in $[Ca^{2+}]_i$ of microglia, and trigger evident outward K^+ currents. Increased $[Ca^{2+}]_i$ level and outward K^+ expression may both act as signals for a functional change in microglia. Therefore, it is reasonable to predict that significant extracellular ATP concentrations can build up under some circumstances in the CNS, stimulate microglial activity and be involved in the pathology of CNS disease.

4. Functional muscarinic receptors in human microglia

The present study demonstrates that human microglia in culture express functional muscarinic receptors which respond to cholinergic neurotransmitters with a Ca^{2+} transient. This transient appeared in more than 50% of the microglia tested and is inhibited by atropine. The CCh-induced $[Ca^{2+}]_i$ response seems to be composed of two parts: an initial $[Ca^{2+}]_i$ spike phase and an elevated plateau phase. The initial $[Ca^{2+}]_i$ transient induced by CCh was also observed in Ca^{2+}-free recording media, however, the elevated plateau phase was not seen. A CCh-induced Ca^{2+} transient has been previously observed in cultured rat microglia, this response was also reported to be dependent on release of Ca^{2+} from intracellular stores (Whittemann et al., 1993). Thus, we suggest that the initial $[Ca^{2+}]_i$ increase results largely from the release of Ca^{2+} from intracellular stores as it is produced in the absence of extracellular Ca^{2+}. We suspect that the plateau component might be

produced by Ca^{2+} influx through the plasma membrane. The functional muscarinic receptors in microglia may be coupled to the inositiol phosphate second messenger system to stimulate intracellular Ca^{2+} store release as reported for muscarinic responses in a variety of other cell types (Buxbaum et al., 1992). A previous study in vascular smooth muscle showed that during agonist stimulation the entry of Ca^{2+} from the external environment plays an important role in maintaining the plateau phase and this plateau phase disappears in Ca^{2+}-free medium (Cauvin & Van Breemen, 1985). Other studies also indicate that the initial increase of $[Ca^{2+}]_i$ is transient, but that the decline does not fall to the resting level in the presence of external Ca^{2+} (Merrit & Rink, 1987; Shieh et al., 1991). Therefore, other mechanisms may operate during the prolonged response. Putney (1986) proposed that the two phases of increase in $[Ca^{2+}]_i$ following receptor stimulation may be intimately linked: IP_3 first stimulates the release of Ca^{2+} from the internal stores and the depletion of these stores then becomes the signal for the entry of Ca^{2+} from outside the cell.

In addition, successive CCh applications produced a second Ca^{2+} response with less amplitude suggesting the receptor desensitization; which might also due to the involvement of cytosolic second messenger in the $[Ca^{2+}]_i$ mobilization, because the successive IP_3 producing need more time to form and accumulate for the IP_3R activation._ The observation that successive CCh applications can not induce a second $[Ca^{2+}]_i$ increase in Ca^{2+}-free media, implies that repeated CCh responses are dependent on the presence of extracellular Ca^{2+} and the lack of internal store replenishment with Ca^{2+} failed to lead to Ca^{2+} release upon receptor stimulation.

The importance of changes in $[Ca^{2+}]_i$ for the regulation of many aspects of cell function is now well established. The rapid changes in $[Ca^{2+}]_i$ of microglia in response to cholinergic stimulation may serve as a second messenger to trigger one or more downstream cascades which have physiological consequences. We know that activated microglia are observed in some

neurodegenerative diseases and may play a role in the initiation or progression of these diseases. Pathologically, senile plaques are associated with degeneration of nearby neuronal cell processes. Microglia are often found on, or around, amyloid plaques and appear to be in proximity to cholinergic terminals (Selkoe, 1991; Pow et al., 1989). It is possible that abnormal cholinergic stimulation of microglia associated with senile plaques results in the activation of microglia and elevations of $[Ca^{2+}]_i$, since ACh is widely distributed in the CNS, and certain neurodegenerative diseases, like dementia or AD are associated with abnormalities in cholinergic pathways.

Amyloid precursor protein (β-APP) is a highly conserved secretory glycoprotein that is expressed at high levels in mammalian brain by neurons, astrocytes, and activated microglia. β-amyloid is an abnormal cleavage product of APP, and the Aβ deposit is thought to be secondary to an overproduction of β-APP. The regulation of β-APP secretion occurs through a complex mechanism that involves activation of various cell surface receptors coupled to protein kinases. Some *in vitro* experiments have demonstrated that β-APP production and processing can be regulated by cholinergic muscarinic receptor ligands such as carbachol and that β-APP secretion is accelerated by agonists for M1 or M3 muscarinic receptors (Nitsch et al., 1992). In addition, protein kinase C (PKC) has been reported to increase secretion of APP in some cell types (Nitsch et al., 1992; Wallace et al., 1993); and PKC was also reported to increase $[Ca^{2+}]_i$ in cultured human microglia (Yoo et al., 1996). All these observations, together with our results showing cholinergic agonists increase $[Ca^{2+}]_i$ suggest that as human microglia generate a Ca^{2+} transient in response to carbachol and acetylcholine, cholinergic stimulation, PKC activation, and increased $[Ca^{2+}]_i$ may be involved in the regulation of β-APP secretion by microglia. However, contrary observations have been obtained from animal studies showing that lesions of cholinergic basal forebrain result in up-regulation of β-APP in the cerebral cortex. A systemically-administrated muscarinic receptor

ligand can affect the in vivo metabolism of β-APP and blockade of muscarinic receptors (chronic atropine treatment) elevates cortical β-APP mRNA levels in the intact animal (Beach et al., 1996). Beach et al have hypothesized that Aβ is accumulated in normal aging as a result of age-related cholinergic terminal loss causing altered regulation of β-APP metabolism.

In addition, muscarinic receptors expressed by microglia might also modulate or fine tune the physiological properties of microglia. That means the activation of AChR could be an additional signaling substance leading to physiological changes in the properties of microglia such as the transition from a resting state to a functional state.

5. Capacitative Ca^{2+} entry in microglia

In the present study, a consistent finding was that when Ca^{2+} was replaced after experiments were performed in Ca^{2+}-free media, microglia always underwent a rapid $[Ca^{2+}]_i$ increase. Similar protocols have been used in human leukemia cells (HL60) (Gardner et al., 1997) and mouse microglia (Ferrari et al, 1996) previously and have shown similar results. Ferrari et al (1996) explained this phenomena as being due to the fact that the P_2X receptor did not inactivate for several minutes after extracellular ATP stimulation. Gardner et al (1997) suggested that this observation occurred because of a store-depletion-operated channel (SOC) mediated Ca^{2+} influx across plasma membrane, also termed capacitative Ca^{2+} entry. Our results support the mechanism of capacitative Ca^{2+} entry since this rapid $[Ca^{2+}]_i$ increase in microglia after Ca^{2+} replacement also occurred with muscarinic receptor activation, or even after Ca^{2+} replacement following incubation in Ca^{2+}-free HBSS for a few minutes. We do not know whether both ATP and ACh stimulated Ca^{2+} entry occurs only through store depletion, or through a receptor stimulation-coupled ion channel, or if there is another alternative coupling mechanism.

ATP- or CCh-induced $[Ca^{2+}]_i$ increases partly resulted from intracellular Ca^{2+} store release; Ca^{2+}-free medium incubation, either with longer or short periods, both decreased the basal $[Ca^{2+}]_i$ level. All of these stimuli may elicit internal Ca^{2+} store depletion. Thus, any one, or a combination of these manipulations could conceivably participate in increasing $[Ca^{2+}]_i$ levels through the mechanism of capacitative Ca^{2+} entry when external Ca^{2+} is added back to the buffer. In addition, a Tg-induced $[Ca^{2+}]_i$ increase, which is also the result of internal store depletion, triggered an elevated and sustained $[Ca^{2+}]_i$ level in Ca^{2+}-containing media, but not in Ca^{2+}-free media. Replacement of external Ca^{2+} under the latter conditions evoked a large $[Ca^{2+}]_i$ increase. This further supports SOC-operated capacitative Ca^{2+} entry in microglia.

The major signal for this Ca^{2+} entry is the loss of Ca^{2+} from $InsP_3$-sensitive stores, which can be initiated by agonist stimulation or inhibition of ER/SR Ca^{2+} ATP-ase. We suggest that a reduction in Ca^{2+} from $InsP_3$-sensitive stores and the upregulation of Ca^{2+} influx mediated by SOC are likely to play major roles in the increased $[Ca^{2+}]_i$ following ATP and CCh treatment of microglia. Apparently, the amplitude of Ca^{2+} release from internal stores induced by ATP, CCh and Tg is different in the present results. This could be because the different agonists triggered different functional parts of the ER, or because of their different dose-dependent effects on ER. Based on these observations, it is possible that the store-operated capacitative Ca^{2+} influx will occur before the whole stores are totally emptied, and that the activation of InsP_3R itself might be more important. This may support the conformational coupling model: the activated $InsP_3R$ is coupled to the SOC receptor, and through their protein-protein interactions serve to increase Ca^{2+} influx. Other explanations for this process is that the influx of external Ca^{2+} seemed to be regulated by the Ca^{2+} content of a portion of ER lying close to the plasma membrane (Putney, 1986). However, the effects of Tg to deplete the store, and the effects of a Ca^{2+}-free incubation induced store depletion

may not act through the InsP_3 second messenger pathway. In this regard, the Ca^{2+} store depletion induces the formation of diffusible Ca^{2+}-influx factor (CIF), which then stimulates Ca^{2+} influx via Ca^{2+} channels on the plasma membrane.

Although a voltage-independent Ca^{2+} entry mechanism, through Ca^{2+}-permeant channels may also serve Ca^{2+} influx, up to now, none of these putative ion channels have been purified or cloned. The most well-established pathway in this regard is the calcium-release-activated or depletion-activated calcium channel (I_{CRAC}) (Zweifach and Lewis, 1993). I_{CRAC} has an extremely low unitary conductance, and it is usually activated by experimental procedures that result in the depletion of stores. This channel is blocked by trivalent and bivalent cations (Hoth and Penner, 1993). For the present study, no ROC blockers like Ni^{2+} or SKF96365 were used to investigate the properties of this Ca^{2+} entry pathway.

Microglia elicited a large $[Ca^{2+}]_i$ increase when external Ca^{2+} was replaced after exposure to Ca^{2+}-free buffer. As discussed earlier, the calcium-sensing receptor (CaR) serves to sense the changes in extracellular Ca^{2+} . No effects were produced in the present study by the use of the CaR agonist, neomycin (50 μM). We did not investigate the effects of other CaR agonists further and can not conclude whether CaR exists in cultured human microglia.

There is growing awareness that capacitative Ca^{2+} entry plays a central role in many aspects of cell signalling. In some cells, such as lymphocytes and *Drosophila* photoreceptors, it can generate signals directly, whereas in other cells it serves to replenish the internal stores especially under conditions where these stores are generating repetitive calcium spikes. Although the IP_3-linked release of Ca^{2+} is only transient, $[Ca^{2+}]_i$ increases are prolonged substantially by capacitative Ca^{2+} entry mechanisms. Thus, besides simply replenishing Ca^{2+} stores, capacitative Ca^{2+} entry undoubtedly plays a physiological role.

Chapter Five CONCLUSIONS

The present observations indicate that exposure of cultured human microglia to ATP is associated with elevations in $[Ca^{2+}]_i$. This $[Ca^{2+}]_i$ increase is a rapid transient, and mediated by both the release of Ca^{2+} from intracellular stores and transmembrane Ca^{2+} influx. ATP induced-release of Ca^{2+} from intracellular stores in microglia appears to be evoked via InsP_3Rs receptors through activation of metabotropic purinoceptors (P_2y). This Ca^{2+} release was followed by Ca^{2+} influx, which might be mediated by ionotropic purinoceptors (P_2x).

ATP application to microglia can produce a depolarizing inward current with a reversal potential close to 0 mV, indicating the opening of non-selective cation pores which might be related to the activation of the P_2x_7/P_2z receptor. ATP can also produce a significant outwardly rectifying K^+ current which is not present under control conditions. The activation of the outward K^+ channel, together with a large membrane depolarization might be involved in microglial transition from a resting state to a functional state. Large amounts of extracellular ATP signal pathological events like ischemia and injury, and this transmitter may be the molecular link between injured neurons and microglia. The present results show that extracellular ATP can induce a transient increase in $[Ca^{2+}]_i$ of microglia, and trigger evident outward K^+ currents. We suspect that significant extracellular ATP concentrations can build up under some circumstances in the CNS, stimulate microglial activity and be involved in the pathology of CNS disease.

Applications of the neurotransmitte ACh and its agonist CCh induced a rapid transient $[Ca^{2+}]_i$ increase in cultured human microglia. The transient increases in $[Ca^{2+}]_i$ are mediated by release of Ca^{2+} from intracellular stores and are followed by Ca^{2+} influx. The carbachol-induced $[Ca^{2+}]_i$ increase is atropine-sensitive indicating that it is acting through the stimulation of

muscarinic receptors. Both ATP- and CCh-induced $[Ca^{2+}]_i$ responses appeared to desensitize upon repeated stimulation. Activation of AChR could be an additional signaling pathway leading to physiological changes in the properties of microglia such as the transition from a resting state to a functional state. It is possible that abnormal cholinergic stimulation of microglia associated with senile plaques results in the activation of microglia and elevations of $[Ca^{2+}]_i$; PKC has been reported to increase secretion of APP in some cell types and PKC was also reported to increase $[Ca^{2+}]_i$ in cultured human microglia. All these observations, together with our results showing cholinergic agonists increase $[Ca^{2+}]_i$ suggest that as human microglia generate a Ca^{2+} transient in response to carbachol and acetylcholine, cholinergic stimulation, PKC activation, and increased $[Ca^{2+}]_i$ may be involved in the regulation of β-APP secretion by microglia.

Capacitative Ca^{2+} entry mechanisms likely exist in human microglia. Release of Ca^{2+} from intracellular Ca^{2+} stores evoked by ATP and CCh were both followed by a large increase in $[Ca^{2+}]_i$ when external Ca^{2+} was replaced in the recording bath. Thus, we suggest that a reduction in Ca^{2+} from $InsP_3$-sensitive stores and the upregulation of Ca^{2+} influx mediated by SOC likely play major roles in the increased $[Ca^{2+}]_i$ following ATP and CCh treatment of microglia. In addition, store depletion occurs in response to SERCA inhibitors, like Tg, or simply by incubating the microglia in Ca^{2+}-free media. Under these two situations a large $[Ca^{2+}]_i$ increase is seen when extracellular Ca^{2+} is reintroduced to the cells. It is reasonable to believe that besides simply replenishing Ca^{2+} stores, capacitative Ca^{2+} entry undoubtedly plays a physiological role.

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