

**EFFECTS OF SELECTED TRANSMITTERS ON FREE CYTOSOLIC
CALCIUM CONCENTRATION AND PYRUVATE DEHYDROGENATION
IN PRIMARY CULTURES OF MOUSE ASTROCYTES**

**Submitted to the Faculty of Graduate Studies and Research
in Partial Fulfillment of the Requirements
for the Degree of Philosophy
in the
Department of Pharmacology
University of Saskatchewan
Saskatoon
Canada**

**by
Ye Chen
1995**

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of the requirements for the

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By

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EFFECTS OF SELECTED TRANSMITTERS ON FREE CYTOSOLIC CALCIUM CONCENTRATION AND PYRUVATE DEHYDROGENATION IN PRIMARY CULTURES OF MOUSE ASTROCYTES

Selected adrenergic, serotonergic, purinergic and peptidergic agonists were chosen to study the effects of neurotransmitters on $[Ca^{2+}]_i$ and pyruvate oxidative metabolism in cultured astrocytes. Experiments were conducted using the fluorescent calcium indicator, indo-1/AM, to examine the changes in $[Ca^{2+}]_i$ and $[1-^{14}C]$ pyruvate to measure the rate of CO_2 formation from pyruvate dehydrogenation.

Noradrenaline induced an increase in $[Ca^{2+}]_i$ via activation of both α_1 and α_2 -adrenergic receptors. The increase in $[Ca^{2+}]_i$ was attenuated by inclusion of phentolamine or yohimbine. Noradrenaline also enhanced dehydrogenation of pyruvate to acetyl-CoA. Clonidine exerted a stimulation on pyruvate dehydrogenation and there was a tendency towards a small stimulation by phenylephrine. Noradrenaline-induced stimulation of flux from pyruvate to acetylCoA was abolished in the calcium-free medium. Exposure to dexmedetomidine led to a biphasic increase in $[Ca^{2+}]_i$ and in pyruvate oxidation. The effect was inhibited both by yohimbine and idazoxan. Chronic treatment with 1 mM lithium chloride decreased noradrenaline induced increase in $[Ca^{2+}]_i$.

Serotonin caused an increase of $[Ca^{2+}]_i$ in concentrations between 10 pM and 10 μ M in dBcAMP treated astrocytes by stimulating 5-HT_{2C} and/or 5-HT_{2A} receptors. Micromolar concentrations of serotonin were required for activation of the 5-HT_{2A} receptor, whereas low nanomolar concentrations stimulated the 5-HT_{2C} receptor. Fluoxetine exerted an agonist effect on $[Ca^{2+}]_i$ due to the stimulation of 5-HT_{2C} receptors. Chronic administration of fluoxetine led to a down-regulation of the response to fluoxetine and to nanomolar concentration of serotonin. Serotonin had no effect on pyruvate dehydrogenation, whereas fluoxetine decreased the formation of CO_2 .

Adenosine and guanosine evoked significant increases in $[Ca^{2+}]_i$, an effect that was not blocked by P_1 antagonists. Vasopressin-induced increase in $[Ca^{2+}]_i$ could be abolished by a V_1 -selective antagonist and not by removal of extracellular Ca^{2+} . Vasopressin failed to affect pyruvate oxidation.

The findings that selected neurotransmitters cause an increase in $[Ca^{2+}]_i$ through specific receptors provides further evidence that astrocytes are targets for many transmitters released from neurons. One possible mechanism, the stimulation of pyruvate dehydrogenation by adrenergic agonists due to an increase in mitochondrial calcium concentration, secondary to the increase in $[Ca^{2+}]_i$, is proposed.



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CERTIFICATION OF THESIS WORK

We, the undersigned, certify that Ye CHEN, candidate for the degree of Doctor of Philosophy has presented a thesis with the following title: *"Effects of Selected Transmitters on Free Cytosolic Calcium Concentration and Pyruvate Dehydrogenation in Primary Cultures of Mouse Astrocytes."* We consider that the thesis is acceptable in form and content, and that a satisfactory knowledge of the field covered by the thesis was demonstrated by the candidate through an oral examination held on December 8, 1995.

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ABSTRACT

Astrocytes express a wide variety of receptors, which are involved in calcium signalling. Elevation of cytosolic free calcium ion concentration ($[Ca^{2+}]_i$) in astrocytes triggers changes in many aspects of CNS functions, including energy metabolism. Noradrenaline induced increases in energy metabolism in muscle has been attributed to its ability to raise mitochondrial Ca^{2+} subsequent to elevated $[Ca^{2+}]_i$ due to receptor activation. However, this phenomenon has not been investigated in astrocytes.

To study the effects of neurotransmitters on $[Ca^{2+}]_i$ and oxidative metabolism of pyruvate, selected adrenergic, serotonergic, purinergic and peptidergic agonists were chosen. Experiments were conducted using the fluorescent calcium indicator, indo-1/AM, to examine changes in $[Ca^{2+}]_i$ and $[1-^{14}C]$ pyruvate to measure rate of labeled CO_2 formation from pyruvate dehydrogenation.

The adrenergic agonist noradrenaline induced an increase in $[Ca^{2+}]_i$ via activation of both α_1 and α_2 receptors as both phenylephrine (α_1 agonist) and clonidine (α_2 agonist) caused an elevation in $[Ca^{2+}]_i$ level. The increase in $[Ca^{2+}]_i$ evoked by either noradrenaline or phenylephrine was inhibited by inclusion of phentolamine (a nonspecific α -antagonist). Yohimbine, an α_2 -antagonist, inhibited both noradrenaline- and clonidine-evoked increases in $[Ca^{2+}]_i$, indicating the existence of a population of α_2 -adrenoceptors in cultured astrocytes. Noradrenaline also enhanced dehydrogenation of pyruvate to acetyl-CoA. Clonidine exerted a stimulation on pyruvate dehydrogenation and there was a tendency towards a small stimulation by phenylephrine. In contrast, CO_2 formation was not increased when the potassium concentration was raised above 5 mM. Noradrenaline-induced stimulation of flux from pyruvate to acetylCoA was abolished in the absence of extracellular calcium (combined with an elevation of the magnesium concentration or a calcium chelator, EGTA), suggesting that the effect is calcium-dependent.

Exposure to dexmedetomidine, a highly specific α_2 -adrenergic agonist, led to a biphasic increase in $[Ca^{2+}]_i$ and in pyruvate oxidation. The effect was inhibited both by yohimbine and idazoxan.

Chronic treatment with 1 mM lithium chloride decreased noradrenaline-induced increase in $[Ca^{2+}]_i$. This is in agreement with the assumption that lithium impairs the turnover in the inositol phosphate (IP) cycle.

Serotonin caused an increase of $[Ca^{2+}]_i$ in concentrations between 10 pM and 10 μ M in dibutyryl cyclic adenosine 3',5'-monophosphate (dBcAMP) treated astrocytes by stimulating 5HT_{2C} and/or 5-HT_{2A} receptors. Micromolar concentrations of serotonin were required for activation of the 5-HT_{2A} receptor, whereas low nanomolar concentrations stimulated the 5-HT_{2C} receptor. Fluoxetine, an antidepressant acting like 5-HT, exerted an agonist effect on $[Ca^{2+}]_i$ in astrocytes. The effect appeared to be due to the stimulation of 5HT_{2C} receptors. Chronic administration of 10 μ M fluoxetine led to down-regulation of the response to fluoxetine and to nanomolar concentration of serotonin but had little, if any, effect on the response to 10 μ M serotonin. Serotonin had no effect on pyruvate dehydrogenation, whereas fluoxetine decreased the formation of CO₂ from [1-¹⁴C]pyruvate.

Adenosine and guanosine, purinergic agonists, evoked significant increases in $[Ca^{2+}]_i$, an effect that was not blocked by P₁ antagonists. Arginine vasopressin (AVP) induced increases in $[Ca^{2+}]_i$ which could be abolished by a V₁-selective antagonist but not by removal of extracellular Ca²⁺. However, AVP failed to affect pyruvate oxidation in astrocytes.

The findings that selected noradrenergic, serotonergic, purinergic and peptidergic agonists cause an increase in $[Ca^{2+}]_i$ in astrocytes through specific receptors provides further evidence that astrocytes are targets for many transmitters released from neurons. One possible mechanism, stimulation of pyruvate dehydrogenation by

adrenergic agonists in astrocytes due to an increase in mitochondrial calcium concentration, secondary to the increase in $[\text{Ca}^{2+}]_i$, is proposed.

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LIST OF ABBREVIATIONS

5-HT:	Serotonin.
α -KG:	α -ketoglutarate.
Acetyl-CoA:	Acetyl coenzyme A.
AMP:	Adenosine monophosphate.
ADP:	Adenosine diphosphate.
ATP:	Adenosine triphosphate.
AVP:	Vasopressin.
Ca ²⁺ :	Calcium.
cAMP:	Cyclic adenosine monophosphate.
CNS:	Central nervous system.
DAG:	Diacylglycerol.
dBcAMP:	Dibutyl cyclic adenosine 3',5'-monophosphate.
Dex:	Dexmedetomidine.
DMSO:	Dimethyl sulfoxide.
EGTA:	Ethyleneglycol-bis-(β -Aminoethyl ether) N,N,N',N'-tetraacetic acid.
GABA:	Gamma-amino butyric acid
GFAP:	Glial fibrillary acidic protein
HEPES:	N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid).
Indo-1/AM:	Indo-1 acetoxymethyl ester.
IP:	Inositol phosphate.
IP ₃ :	Inositol-1,4,5-triphosphate.
IP ₄ :	Inositol-1,3,4,5-tetrakisphosphate.
K _d :	Dissociation constant (for indo-1).
NA:	Noradrenaline.
NMDA:	N-methyl-D-aspartate.

PBS:	Phosphate buffered saline.
PDH:	Pyruvate dehydrogenase.
PDHa:	Active pyruvate dehydrogenase.
PI:	Phosphatidylinositol.
PIP ₂ :	Phosphatidylinositol-4,5-bisphosphate.
PKC:	Protein Kinase C.
PLC:	Phospholipase C.
R:	Ratio of fluorescence intensities at 405 nm to 485 nm emission wavelength.
R _{max} :	Maximal ratio of fluorescence intensities at 405 nm to 485 nm emission wavelength.
R _{min} :	Minimal ratio of fluorescence intensities at 405 nm to 485 nm emission wavelength.
TCA:	Tricarboxylic acid.
[Ca ²⁺] _i :	Free cytosolic calcium concentration.

1. INTRODUCTION

Astrocytes are now recognized as a class of cells with many important and diverse functions. Astrocytes are up to 10-fold more numerous than neurons in the mammalian central nervous system and can comprise up to 50% of the total cell volume (Pope, 1978). Their processes surround capillaries and synapses, form a continuous subpial and subependymal layer, invest otherwise free neuronal surfaces, and form a syncytium with other astrocytes through gap junctions (Peters et al., 1976). It has been known that astrocytes are involved in the regulation of ion homeostasis at the cellular level (e.g. of potassium, calcium and hydrogen ions), in the accumulation and metabolism of some amino acid transmitters and metabolic substrates and in the responses to a multitude of neurotransmitters (Juurink and Hertz, 1992). It is now evident that the brain cannot be fully understood without an understanding of the many roles of astrocytes.

1.1. Morphological aspects of astrocytes

1.1.1. Types of astrocytes

The brain of mammals consists of neurons, glial cells, and cells belonging to the vasculature. Glial cells are classified into two groups: macroglia and microglia. The macroglia have been divided into astrocytes and oligodendrocytes. Astrocytes come in two main forms: the fibrous astrocyte is found in white matter and the protoplasmic astrocyte in grey matter (Peters et al., 1976).

Within the central nervous system (CNS), glial fibrillary acidic protein (GFAP) is an unequivocal marker for astrocytes (Eng et al, 1971). Primary neuroglial cultures

prepared from perinatal rodent brain contain two types of GFAP-positive astrocytes: type 1 and type 2 astrocytes (Juurlink and Hertz, 1992). Although they both are GFAP positive, they differ in a number of morphological and functional properties. They are derived from two distinct developmental lineages. Type 1 astrocytes develop from type-1 astroblasts, whereas type-2 astrocytes are derived from a common bipotential O2A progenitor cell. Depending on culture environment, O2A progenitors can be induced to differentiate into either oligodendrocytes or type-2 astrocytes. Most type-1 astrocytes in neuron-free cultures are large, flat, non-process-bearing, GQ1C ganglioside-negative, GFAP-positive cells. Type 2 astrocytes are GQ1C ganglioside-positive, smaller, process-bearing cells, and in culture bind both tetanus toxin and A2B5 (Eisenbarth et al., 1979). Functionally, type-1 astrocytes form the glial limiting membrane, and may play a constitutive role in the formation of the blood-brain barrier. McCarthy et al. (1988) have shown β -adrenoceptor binding sites on type-1 but not type-2 astrocytes. Cultured type-2 astrocytes avidly accumulate [^3H] GABA, via a transport system sensitive to several inhibitors of GABA uptake (Levi et al., 1983). In contrast to type-1 astrocytes, type-2 astrocytes don't readily metabolize GABA but will release it upon stimulation with glutamate (Gallo et al., 1989). Also, a much greater density of sodium channels are found in type-2 than type-1 astrocytes. It is unknown whether type-2 astrocytes exist in vivo (Fulton et al., 1991). Type-1 astrocytes correspond to astrocytes in the CNS in situ.

1.1.2. Heterogeneity of astrocytes

Primary cultured astrocytes have different properties according to their source in the brain (Hansson, 1988). Astrocytes cultured from anatomically distinct brain regions exhibit markedly different neuronal support capabilities (Chamak et al., 1987) and differ in the uptake and metabolism of neurotransmitters (Drejer et al., 1982; Schousboe and Divac, 1979), in the electrophysiological properties, and in the expression of receptors

(Hansson et al., 1984), neuropeptides (Shinoda et al., 1989), enzymes (Hansson, 1984), cytoskeletal elements (Papasozomenos and Binser, 1986), and surface glycoproteins (Barbin et al., 1988).

Recent studies have also shown that astrocytes from the same regions are composed of different subpopulations expressing distinct neuroligand receptors. For example, histamine induced responses in about half of the astrocytes in explant cultures of the brain stem and spinal cord in an electrophysiological study (Hosli et al., 1984). An autoradiographic binding study showed that about 66% of polygonal astrocytes expressed α_1 -adrenergic receptors (Lerea and McCarthy, 1989). Serotonin evoked a Ca^{2+} response in up to 80% of astrocytes rat cerebral cortex (Nilsson et al., 1991a).

The concept of regional heterogeneity in astrocytes suggests that (1) neuronal-astroglial interactions are brain region specific; (2) astrocytes might serve special functions in different areas of the brain; (3) conclusions based on single region studies may not apply to other regions of the CNS (Wilkin et al., 1990).

1.1.3. Effect of dibutyryl cyclic AMP on cultured astrocytes

In general, astrocytes develop ontogenetically later than neurons (Brizzee and Jacobs, 1959a,b; Brizzee et al., 1964). In vivo, cerebral cortical astrocytes proliferate and differentiate mainly at post-natal stage in both rat and mouse (Privat, 1975). Primary cultures of astrocytes prepared from newborn mice contain no neurons, which means that cultured astrocytes do not receive noradrenergic signals. This is in contrast to astrocytes in vivo where noradrenergic innervation begins at embryonic day 16, and the development of the neocortical fibers continues through the first 2 postnatal weeks (Foote et al., 1983). This may imply that cultured astrocytes morphologically and

functionally remain undifferentiated unless this lack of noradrenergic innervation is compensated for (Hertz, 1990a).

By including dibutyryl cyclic AMP (dBcAMP), a lipid-soluble cAMP derivative, in the culturing medium to increase the intracellular level of cAMP, one is able to mimic adrenergic induction of a morphological transformation of astrocytes from a flat, polygonal cell shape into a process-bearing, stellate cell type (Shapiro, 1973; Lim et al., 1973). This morphological transformation, often designated as differentiation, is accompanied by marked biochemical changes. The content of GFAP is enhanced (Hertz et al., 1978). The rates of oxygen consumption and of glutamate oxidation are increased (Hertz and Hertz, 1979; Hertz, 1990a). The activities of MAO_B and Na⁺-K⁺ATPase rise (Schousboe et al., 1976). The removal efficacy of GABA from the extracellular space is enhanced. The voltage dependent L calcium channel is induced (MacVicar, 1984; Hertz et al., 1989a), and the expression of cell surface receptors is altered (McCarthy et al., 1988). All of these alterations might be essential for astrocytic functions in the adult CNS.

1.2. Functional aspects of astrocytes

1.2.1. Receptors for neurotransmitters on astrocytes

Astrocytes are not excitable cells (Berwald-Netter et al., 1986), but from biochemical, histochemical and electrophysiological studies there is strong evidence that astrocytes express receptors for a variety of neurotransmitters and neuromodulators (Hertz et al., 1984; Hosli and Hosli, 1988a; Kimelberg, 1988). After release from neurons, these neurotransmitters act on astrocyte receptors to mediate increases of intracellular concentrations of diacylglycerol, inositol triphosphate, Ca²⁺, cAMP and

cGMP, which can, in turn, modulate the activity of ion channels and certain enzymes, regulate energy metabolism, e.g., by mobilizing glycogen stores, or participate in astrocyte volume regulation (Murphy and Pearce, 1987; Sorg and Magistretti, 1991; Huang et al., 1994b). Since the neurotransmitters are released from neurons, the effects on astrocytes represent a neuronal-astrocytic interaction. Many of the actions on astrocytes will, in turn, influence neuronal functions (Hertz and Peng, 1992a). In the following, receptor characteristics of noradrenaline, serotonin, purines and the peptide hormone, vasopressin, will be briefly reviewed.

1.2.1.1. Noradrenaline

A great number of studies have shown that astrocytes express both α - and β -adrenergic receptors (Hosli and Hosli, 1982; Hertz et al., 1984; Bockaert and Ebersolt, 1988). Histochemical studies have demonstrated that astrocytes possess β_1 -adrenergic receptors (Aoki et al., 1987; Stone et al., 1992).

In addition to the β -adrenergic receptors, astrocytes in culture also express α -adrenergic receptors. Up to now, four different α_1 -adrenergic subtypes have been identified (α_{1A} , α_{1B} , α_{1C} and α_{1D}). The two subtypes α_{1A} and α_{1B} have been shown to be present on astrocytes in vitro (Wilson et al., 1990). The two receptor subtypes can be pharmacologically distinguished by their different affinities for the competitive antagonist WB 4101 and their different sensitivity to alkylation by chlorethylclonidine. Functionally, the α_{1A} receptor is thought to predominantly mediate Ca^{2+} influx from the extracellular environment, while activation of the α_{1B} receptor results in phosphoinositide (PI) breakdown and subsequent release of Ca^{2+} from intracellular stores (Bylund, 1992).

It was originally assumed that all α_2 -adrenergic receptors are located presynaptically and inhibit noradrenaline release (Starke et al., 1975; Weiss et al., 1986), but during the last 10 - 15 years compelling evidence of an additional localization on target cells has appeared (Unnerstall et al., 1984). α_2 -Adrenergic receptors are found on astrocytes (Bockaert and Ebersolt, 1988). Three subtypes of the α_2 -adrenergic receptors have been identified (α_{2A} , α_{2B} , α_{2C}) (Bylund, 1992). Moreover, the so-called imidazoline preferring receptors are present in astrocytes (Regunathan et al., 1993). Imidazole derivatives like the α_2 -adrenergic agonists, clonidine and dexmedetomidine, stimulate not only genuine α_2 -adrenergic receptors but also imidazoline preferring receptors (Bylund, 1992; Venkataraman, 1992).

Autoradiographic studies by Hosli and Hosli (1982) have shown that the number of astrocytes labelled by α -adrenergic antagonists is usually smaller than that labelled by β -adrenergic compounds, suggesting that astrocytes possess more β -adrenergic sites. Furthermore, astrocytes revealed more binding sites for α_1 than α_2 -ligands. Using electrophysiological techniques, astrocyte depolarizations by α -agonists and hyperpolarizations by β -agonists were observed on the same cell, indicating that α - and β - adrenoceptors co-exist on the membrane of the same astrocytes.

Astrocytes respond to noradrenaline or isoproterenol with increases in the intracellular levels of cyclic AMP, glycogenolysis and Na^+ , K^+ - ATPase activity (McCarthy and deVellis, 1978; Hansson et al., 1984; Subbarao and Hertz, 1990; Hajek et al., 1996). Stimulation of α - receptors does not affect basal cAMP levels but inhibits the β - adrenergic- mediated increase in cAMP (Van Calcar et al., 1979), an inhibition which is probably mediated by α_2 - adrenergic receptors.

There is much evidence that activation of adrenergic receptors causes changes in the cytosolic concentration of Ca^{2+} in astrocytes. Investigations by means of the fluorescent calcium indicators, fura-2 and indo-1, have shown that noradrenaline and other adrenergic agonists evoke a rise in intracellular calcium levels of primary astrocytes of rat CNS (Salm and McCarthy, 1990; Delumeau et al., 1991). From pharmacological studies using selective α -adrenergic agonists and antagonists, it was concluded that astroglial Ca^{2+} -responses are mediated by α_1 - and α_2 -adrenergic receptors. Stimulation of α -receptors in primary cultures of astrocytes markedly increase the formation of inositol triphosphate, an action which was mimicked by α_1 - agonists (Pearce et al., 1985; Nilsson et al., 1991b).

Activation of astrocytic α -adrenergic receptors induces glycogenolysis. Glycogenolysis was markedly enhanced by stimulation of either α_2 -or β -adrenoceptors (Subbarao and Hertz, 1990). Stimulation of α_1 -receptors increases lactate formation whereas oxidative metabolism was enhanced by stimulation of both α_1 - and α_2 -adrenoceptors (Subbarao and Hertz, 1991).

Dexmedetomidine, a potent and highly specific α_2 -adrenergic agonist which in receptor binding experiments has an α_2/α_1 selectivity ratio of 1600 (Virtanen, 1989), previously has been found to increase $[\text{Ca}^{2+}]_i$ in astrocytes (Zhao et al., 1992).

1.2.1.2. Serotonin

Biochemical studies have shown that serotonin (5-HT) binds to astrocytes in primary cultures from rat and mouse (Hertz et al., 1979; Tardy et al., 1982). Autoradiographic studies demonstrated that astrocytes express binding sites for ^3H -5-

HT and the 5HT₂-antagonist ³H-ketanserin, suggesting that astrocytes might possess both 5HT₁- and 5HT₂-receptors (Hosli and Hosli, 1987).

Serotonin induces a breakdown of phosphoinositides in astrocytes (Ananth et al., 1987; Hansson et al., 1987). It has been repeatedly observed that exposure to serotonin causes an increase in free cytosolic calcium concentration in astrocytes (Deecher et al., 1993; McCarthy and Salm, 1991; Nilsson et al., 1991a), and it has been concluded that this is due to stimulation of 5-HT_{2A} receptors. This is consistent with the finding that micromolar concentrations of serotonin are required for this response (Deecher et al., 1993; Nilsson et al., 1991b). Serotonin also causes an increase in glycogenolysis in astrocytes (Cambray-Deakin et al., 1988), a response that might be secondary to the increase in cytosolic calcium concentration (Hertz and Code, 1993; Subbarao et al., 1995). The stimulation of the 5-HT_{1A} receptor causes release of S-100, a brain specific protein found in abundance in astrocytes (Hyden and McEwen, 1966). The released S-100 acts as a growth factor for serotonergic neurons and enhances their differentiation (Azmitia et al., 1991).

Fluoxetine is a serotonin uptake inhibitor which is used as an antidepressant. It does, however, also have affinity for the 5-HT_{2C} receptor in cultures of astrocytes (Zhang et al., 1993). The effect of fluoxetine on serotonergic transmission by chronic treatment is unknown.

1.2.1.3. Purines

Two major types of purinergic receptors, P₁ and P₂, have been distinguished (Burnstock, 1978). P₁ receptors are more responsive to adenosine and AMP than to ADP and ATP, and are coupled to adenylate cyclase. P₁ receptors have been subdivided

into A₁ and A₂ receptors according to the relative potencies of adenine analogs and to whether adenylate cyclase activity is increased (A₂) or decreased (A₁) by these analogs. Biochemical and autoradiographic studies have indicated the existence of both A₁- and A₂-receptors on astrocytes (Van Calcar et al., 1979; Hosli and Hosli, 1988b). At submicromolar concentrations, adenosine was found to inhibit the increase of cAMP by activating A₁-receptors, whereas at micromolar or at higher concentrations, the nucleotide increased the level of cAMP by stimulating A₂-receptors (Van Calcar et al., 1979).

P₂ receptors are more responsive to ATP and ADP than to AMP and adenosine, and their occupation leads to prostaglandin synthesis while cAMP levels are unaffected. P₂ receptors have been subdivided into P_{2X} and P_{2Y} types according to relative potencies of ATP analogs.

In astrocytes, adenosine, ADP and ATP stimulate Ca²⁺ influx as well as inositol trisphosphate formation and Ca²⁺ mobilization (Neary et al., 1988; Pearce et al., 1989; Kastritsis et al., 1992). Adenosine was also found to elicit hydrolysis of glycogen in cultured astrocytes from rat brain (Magistretti et al., 1983). ATP stimulates prostaglandin synthesis which is a response mediated by P_{2Y} receptors (Bruner and Murphy, 1990).

1.2.1.4. Peptide transmitters

The only peptide transmitter studied was vasopressin. Vasopressin (AVP) released into the systemic circulation from the pituitary does not easily cross the blood-brain barrier (Ang and Jenkins, 1982), but vasopressin is also released into the brain itself. In addition to the neurosecretory pathways to the pituitary, vasopressin

immunoreactive fibers reach many other areas of the brain. It has been shown that cortical astrocytes express vasopressin receptors (Hosli et al., 1991). Vasopressin has been found to induce an increased turnover of inositide phosphates (i.e., a response characteristic for the V_1 receptor) in astrocytes prepared from the cerebral hemispheres (Cholewinski et al., 1988). A small increase in cell volume of astrocytes in primary cultures that were incubated under steady-state conditions has been observed after exposure to 10^{-5} M vasopressin (Del Bigio and Fedoroff, 1986). Latskovits et al. (1992) found that a 3-hr incubation of primary cultures of astrocytes with 10^{-6} M vasopressin caused a large increase in intracellular water space. Also, an increase of potassium-induced swelling in the presence of vasopressin was observed in primary cultures of astrocytes with a threshold at about 10^{-12} M and an interdependence between the co-transport system for Na^+ , K^+ and 2Cl^- and vasopressin was suggested (Chen et al., 1992). The concept has been experimentally verified by Latskovits et al. (1993) who have demonstrated the increased astrocytic swelling in the presence of vasopressin is abolished in the presence of bumetanide, an inhibitor of the co-transport system. AVP also was found to mobilize astrocytes (pituicytes) intracellular Ca^{2+} in the absence of extracellular Ca^{2+} via V_1 receptors (Hatton et al., 1992).

1.2.2. Calcium signalling in astrocytes

1.2.2.1. Calcium homeostasis

Free intracellular calcium ions serve as a second messenger in astrocytes. The resting free cytosolic calcium concentration is maintained near 10^{-7} M, about four orders of magnitude lower than extracellular calcium concentration (e.g. $> 10^{-3}$ M). Total Ca^{2+} content is estimated to be 10^5 times the amount of free intracellular Ca^{2+} (Williams, 1989). Very high concentrations of Ca^{2+} (≈ 1 -100 mM) are present in internal sources,

known as Ca^{2+} stores or compartments. Therefore, most cellular Ca^{2+} is not free. The mechanisms of calcium homeostasis in astrocytes involve Ca^{2+} entry via cell membrane voltage- and receptor- operated channels and Ca^{2+} release from intracellular stores, while Ca^{2+} removal is brought about by the function of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, plasma membrane Ca^{2+} pumps, and Ca^{2+} -binding proteins.

Sources of cytosolic calcium increase

Transiently increasing the Ca^{2+} permeability of the plasma membrane or releasing Ca^{2+} from internal stores by opening various types of Ca^{2+} channels, leads to the increase of intracellular calcium. Thus, two main sources for stimulus induced intracellular Ca^{2+} increase are extracellular milieu and intracellular stores.

Calcium entry

The movement of Ca^{2+} into the cell causes an inward electrical current. Since the intracellular calcium concentration is much lower than the extracellular concentration, there exists a large diffusion gradient for Ca^{2+} ions into the cell. The membrane is, however, very impermeable to calcium ions, and calcium only enters the cell when channels in the membrane open. At least two types of channels, voltage-dependent calcium channels and receptor-operated calcium channels, regulate Ca^{2+} entry into astrocytes. A third type may be second messenger operated calcium channels.

Voltage-operated calcium channels. According to their biophysical and pharmacological properties, voltage-operated calcium channels can be categorized as being of the L, N, T and P type (Tsien et al, 1988; Miller, 1992). Astrocytes express at least two types of voltage-operated Ca^{2+} channels. L- and T- type currents have been observed in astrocytes (MacVicar, 1984; Barres et al., 1989). Fluorometric techniques

show that these channels mediate sufficient Ca^{2+} influx to significantly increase cytosolic Ca^{2+} in cerebral cortical, hippocampal, and cerebellar astrocytes.

However, L-channels are found in cultured astrocytes treated with dBcAMP but not in untreated cells (MacVicar, 1984; Hertz, 1990). Astrocytes grown in specific batches of serum (Barres et al., 1989) or co-cultured with neurons (Corvalan et al., 1990) also express Ca^{2+} channels. These studies suggest that astrocytic Ca^{2+} channel expression is modulated by environmental factors, such as adenylate cyclase.

Receptor-operated calcium channels. The second major pathway for transmembrane calcium influx is coupled with the activation of agonist-operated channels. Simultaneous patch-clamp and fluorometric measurements have shown that activation of AMPA-kainate sensitive glutamate receptors produced Ca^{2+} influx independently of voltage changes (Müller et al., 1992). Extracellular ATP may cause a Ca^{2+} influx by directly opening a surface membrane Ca^{2+} channels (Neary et al., 1988).

Second messenger operated calcium channels. There is growing evidence that inositol 1,4,5-trisphosphate (IP_3) may directly activate specific channels in the plasma membrane. Olfactory cells possess an IP_3 -sensitive calcium channel (Berridge, 1993). IP_3 -evoked increase in Ca^{2+} influx via activation of Ca^{2+} channels has been demonstrated in lymphocytes (Khan et al., 1992). It is unknown if astrocytes also possess this receptor.

Calcium release

It was first reported by Streb et al. (1983) that inositol-triphosphate can cause calcium release from an intracellular calcium store of pancreatic acinar cells, a finding that was soon after confirmed using other cell types (Berridge, 1984). It has now been demonstrated that there is an accumulation of inositol phosphates in cultured astrocytes in response to a variety of neurotransmitters and hormones (Pearce et al., 1986; Hansson et al., 1987). Stimulation of receptors in astrocytes leads to activation of a membrane-

bound phosphodiesterase called phospholipase C. Activated phospholipase C subsequently cleaves membrane-bound phosphatidylinositol 1,4,5,-trisphosphate, releasing two fragments, inositol 1,4,5,-trisphosphate and diacylglycerol (DAG). IP₃ acts on intracellular calcium stores to liberate calcium into the cytosol, while diacylglycerol stimulates protein kinase C (PKC) in the presence of an elevated intracellular calcium concentration (Berridge, 1987). Activation of PKC regulates a multitude of events on the cell membrane (enhancement or inhibition of ion channel and ion carrier activity) (Rane and Dunlap, 1986; Marchetti and Brown, 1988) and in the cell interior (e.g. protein synthesis and phosphorylation) (Neary et al., 1986; Saunders and DeVries, 1988).

Both IP₃ and DAG are quickly metabolized in the cell. IP₃ can be phosphorylated by IP₃ kinase to form inositol-1,3,4,5-tetrakisphosphate (IP₄) (Irvine et al., 1986; Irvine, 1991) or can be sequentially dephosphorylated to inositol and eventually reform the lipid phosphatidylinositol (PI). IP₄ has been suggested to be involved in entry of Ca²⁺ from outside the cell and in expanding the IP₃ sensitive Ca²⁺ pool (Berridge and Irvine, 1989; Irvine, 1991). DAG can either be phosphorylated to phosphatidic acid (eventually reforming PI) or it can be hydrolysed by a lipase forming arachidonic acid.

Lithium inhibits the conversion of IP₂ to IP₁, an essential step in the normal recycling of membrane phosphoinositides (Bunney and Garland-Bunnery, 1987). This block leads to a depletion of phosphatidylinositol-4,5-bisphosphate (PIP₂), and the effects of transmitters on the cell will diminish in proportion to the amount of activity in the PIP₂-dependent pathways.

IP₃-sensitive Ca²⁺ stores seem to be the predominant internal Ca²⁺ pool in astrocytes. It is not clear if astrocytes possess an intracellular Ca²⁺ store which can undergo Ca²⁺-induced Ca²⁺-release (Finkbeiner, 1993). However, dantrolene was found to partially suppress [Ca²⁺]_i oscillations in astrocytes, while caffeine and

ryanodine were ineffective (Charles et al., 1993). This suggests the absence of a caffeine- and ryanodine- sensitive Ca^{2+} -induced Ca^{2+} -release mechanism in astrocytes.

Removal of calcium from the cytosol

All calcium that enters from the extracellular space or is released from internal stores must eventually be removed to maintain cell functions. Actually, most Ca^{2+} is buffered or sequestered, or extruded in order for the cells to remain in Ca^{2+} balance.

Calcium buffering by calcium-binding protein

In many tissues, such as neurons and muscles, Ca^{2+} entering the cytosol would initially encounter with high-affinity Ca^{2+} -binding proteins. These Ca^{2+} -binding proteins are a variety of soluble proteins all of which bind Ca^{2+} with relatively high affinity (10^{-5} to 10^{-8}M) (Baker and Umbach, 1987; Donahue and Abercrombie, 1987). The presence of Ca^{2+} -binding proteins in the cytosol is important as it plays a role in sequestering free $[\text{Ca}^{2+}]$ in solution much more rapidly than Ca^{2+} pumps can extrude elevated $[\text{Ca}^{2+}]_i$ (Miller, 1991). The cytosolic Ca^{2+} pulse, therefore, has a shortened period in the cell as free calcium but a longer period as unavailable calcium bound to parvalbumin. However, to date very little is known about Ca^{2+} -binding proteins in astrocytes.

Sequestration of calcium in intracellular organelles

Cytosolic Ca^{2+} buffering, although rapid, may be very limited in capacity. In cells that are excited at relatively high frequency, and especially in those in which a relatively large fraction of the inward current during the rising phase of the action potential is carried by Ca^{2+} , the cytosolic buffers may rapidly saturate. Intracellular

organelles are therefore required to sequester Ca^{2+} until the Ca^{2+} load can be extruded across the plasma membrane.

Endoplasmic reticulum. The endoplasmic reticulum probably plays an important role in sequestering Ca^{2+} following cell activity. The properties of the Ca^{2+} uptake system in the sarcoplasmic reticulum of heart, skeletal and smooth muscle have been most extensively studied (Carafoli, 1987). During a train of action potentials, the endoplasmic reticulum will begin to accumulate Ca^{2+} because Ca^{2+} extrusion across the plasma membrane lags behind Ca^{2+} entry (Blaustein, 1988). Most other types of cells, including astrocytes, also contain a sequestration system that has usually been referred to as smooth endoplasmic reticulum. The accumulation of Ca^{2+} into the endoplasmic reticulum is an energy dependent process. A membrane associated Ca^{2+} -ATPase (Ca^{2+} pump) is responsible for this lowering of cytosolic Ca^{2+} . The properties of the Ca^{2+} pump in the endoplasmic reticulum will be discussed below.

Mitochondria. In mitochondria, Ca^{2+} enters and exits by different pathways. Uptake of Ca^{2+} occurs via an electrophoretic uniporter driven by the large electrical potential across the inner membrane. Ca^{2+} uptake can be inhibited physiologically by Mg^{2+} and artificially by ruthenium red and activated by spermine (McCormack and Denton, 1993).

The principal efflux mechanism involves obligatory exchange between Ca^{2+} and Na^{+} with the probable stoichiometry of $2 \text{Na}^{+} : 1 \text{Ca}^{2+}$; this exchange is linked to the chemiosmotic H^{+} circuit via $\text{Na}^{+}:\text{H}^{+}$ exchange. Mitochondria may also contain a Na^{+} -independent mechanism for Ca^{2+} efflux, although it is controversial whether this involves direct $\text{Ca}^{2+}:\text{H}^{+}$ exchange (McCormack et al., 1990).

Role of mitochondrial Ca^{2+} transport under normal cellular conditions: It was previously thought that mitochondria also serve to regulate Ca^{2+} in order to regulate cytosolic Ca^{2+} . However, it is now widely regarded that matrix Ca^{2+} concentration is regulated within the micromolar range, dependent upon changes in cytosolic Ca^{2+}

(McCormack et al., 1990). This is important because mammalian tissues contain three important dehydrogenases within mitochondria. They are pyruvate dehydrogenase, α -oxoglutarate dehydrogenase and NAD-dependent isocitrate dehydrogenase. These mitochondrial enzymes are activated by Ca^{2+} within the physiologic range of intramitochondrial Ca^{2+} (0.1 to 10 μM). The calcium induced changes in enzyme activities could play a central role in the regulation of cell metabolism. Increases in mitochondrial Ca^{2+} via the above mechanisms would occur when cytosolic Ca^{2+} is relatively high and the energy demands are also high. The activation of these enzymes allows increases in ATP production which do not depend upon diminished cellular NADH/NAD⁺ or ATP/ADP concentration ratios. This therefore provides a means to balance the increases in energy demands which are brought about by raised cytosolic Ca^{2+} in its activation of processes such as contraction and secretion.

Role of mitochondrial Ca^{2+} transport under pathophysiological conditions:

Isolated mitochondria can accumulate very large amounts of Ca^{2+} , particularly in the presence of inorganic phosphate due to the precipitation of insoluble Ca^{2+} -phosphate deposits in a process known as matrix loading (Carafoli, 1987). However, in a situation closer to *in vivo* conditions, Fry et al. (1984) showed that mitochondrial Ca^{2+} uptake was not appreciable in hyperpermeabilized isolated cardiac myocytes until cytoplasmic Ca^{2+} exceeded 1 μM . Blaustein and colleagues (1988) demonstrated that application of large "unphysiological" Ca^{2+} loads to saponin permeabilized synaptosomes allowed Ca^{2+} uptake into mitochondria. Thus, it is likely that the Ca^{2+} -transport system plays a key role in certain pathophysiological circumstances (Miller, 1991), such as ischemia and reperfusion, where there appears to be abnormal and high cellular Ca^{2+} influx.

Extrusion of calcium across the plasma membrane

A very large gradient between intracellular and extracellular Ca^{2+} concentration and large inward electrical driving force require that cells must expend metabolic energy

to extrude Ca^{2+} . Astrocytes are believed to have two parallel, independent mechanisms for extruding Ca^{2+} : an ATP-driven Ca^{2+} pump; and a $\text{Na}^+/\text{Ca}^{2+}$ exchanger transport system.

The $\text{Na}^+/\text{Ca}^{2+}$ exchanger couples the translocation of Na^+ in one direction with that of Ca^{2+} in the opposite direction. The major role of the exchanger is to pump Ca^{2+} out of the cell. It has been demonstrated that 3 Na^+ per Ca^{2+} exchange stoichiometry is adequate to drive enough energy from the transmembrane Na^+ gradient to transport Ca^{2+} across the plasma membrane (Carafoli, 1987). The $\text{Na}^+/\text{Ca}^{2+}$ exchanger also mediates net Ca^{2+} influx upon reduction of the Na^+ electrochemical gradient (Goldman, et al., 1994). Immunohistochemistry revealed that exchanger molecules are distributed in a reticular pattern over the astrocytic surface (Goldman, et al., 1994). The presence of $\text{Na}^+/\text{Ca}^{2+}$ exchanger mRNA in astrocytes was confirmed by Northern blot analysis using cardiac $\text{Na}^+/\text{Ca}^{2+}$ exchanger cDNA probe (Takuma et al., 1994).

The Ca^{2+} pump is a specific ATPase which can move Ca^{2+} against its own electrochemical gradient by using the energy of hydrolysis of ATP. Ca^{2+} ATPase is histochemically localized in astrocyte plasma membranes (Maggio et al., 1991). The plasma membrane pumps are structurally and immunologically different from the Ca^{2+} sequestering pumps in the smooth endoplasmic reticulum (Grover and Khan, 1992). A Ca^{2+} pump in the plasma membrane removes Ca^{2+} from the cytosol into the extracellular space while the one in the smooth endoplasmic reticulum can transport Ca^{2+} back to the smooth endoplasmic reticulum.

1.2.2.2. Functional role of $[\text{Ca}^{2+}]_i$

It has become clear that astrocytic free cytosolic calcium concentration is enormously important for the regulation of a variety of processes. These include metabolism, gene expression, cytoskeletal plasticity, neuronal-astrocytic interaction, and

cell death under various pathological conditions (Kostyuk and Verkhratsky, 1994). A raised $[Ca^{2+}]_i$ stimulates glycogenolysis in astrocytes. An enhanced glycogenolysis may increase the ability of the astrocytes themselves to perform energy requiring work and increase neuronal energy metabolism by supplying increased amounts of pyruvate for further oxidative metabolism (Hamprrecht and Dringen, 1994; Hertz and Hertz, 1995). Ca^{2+} rises have been implicated in modulation of astrocytic differentiation, proliferation, DNA synthesis and mRNA transcription. Protein synthesis may depend on Ca^{2+} , since removal of extracellular Ca^{2+} reversibly depresses protein synthesis (Brostrom et al., 1990). Glutamate release from neurons induces an increase in $[Ca^{2+}]_i$ in cultured hippocampal astrocytes (Cornell-Bell et al., 1990), which is capable of spreading in a wave form across long distances in the tissue, suggesting that Ca^{2+} may serve as a signal in intercellular communications among astrocytes and neurons (Cornell-Bell and Finkbeiner, 1991; Nedergaard, 1994). In cultured astrocytes, prolonged glutamate exposure causes swelling and cytoskeletal filament aggregation in a Ca^{2+} -dependent way (Koyama et al., 1991). CNS diseases may damage astrocytes by disturbing Ca^{2+} homeostasis. Astroglia lose their ability to regulate Ca^{2+} properly during hypoxia and upon reperfusion become Ca^{2+} overloaded (Kim-Lee et al., 1992).

1.2.3. Pyruvate metabolism in astrocytes

1.2.3.1. Pathways of pyruvate metabolism

The metabolism of pyruvate is an especially pivotal step in glucose degradation. Pyruvate is formed by glycolytic degradation of glucose. It can be converted to lactate or further metabolized in the tricarboxylic acid (TCA) cycle. Under aerobic conditions, pyruvate can undergo an oxidative decarboxylation to form acetyl coenzyme A (acetyl-CoA). Acetyl-CoA is condensed with oxaloacetate to form citrate, which is reconverted

to oxaloacetate by one turn of the cycle, resulting in the production of CO_2 , water, ATP and no net synthesis of any TCA cycle intermediate (Fig. 1.1.).

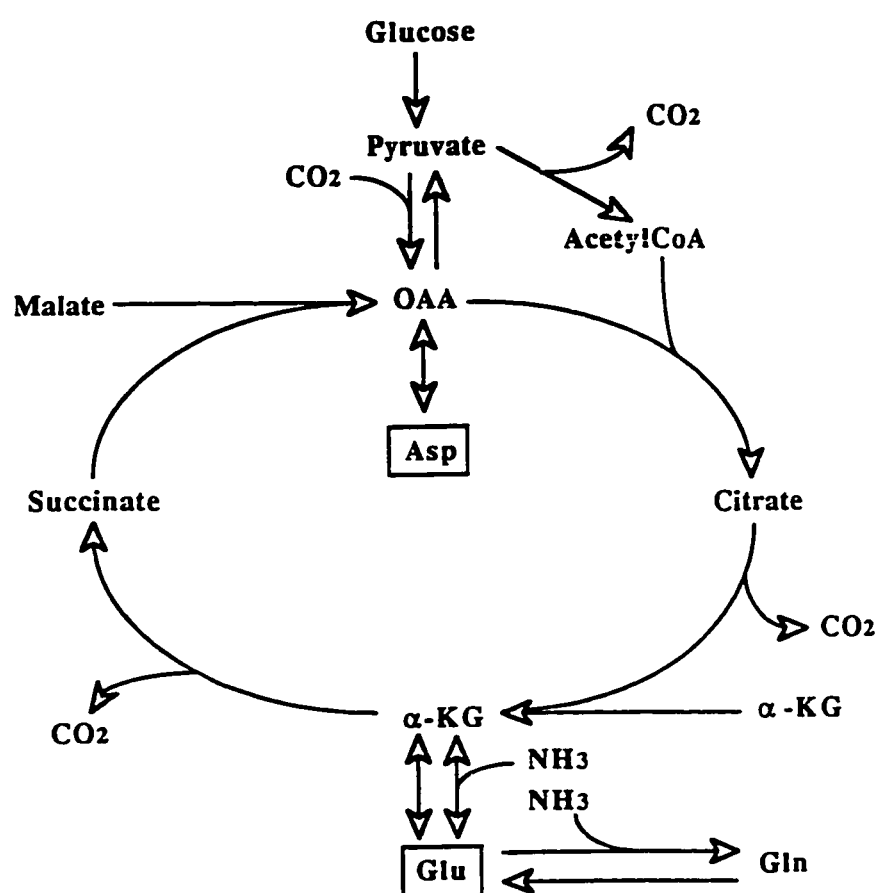
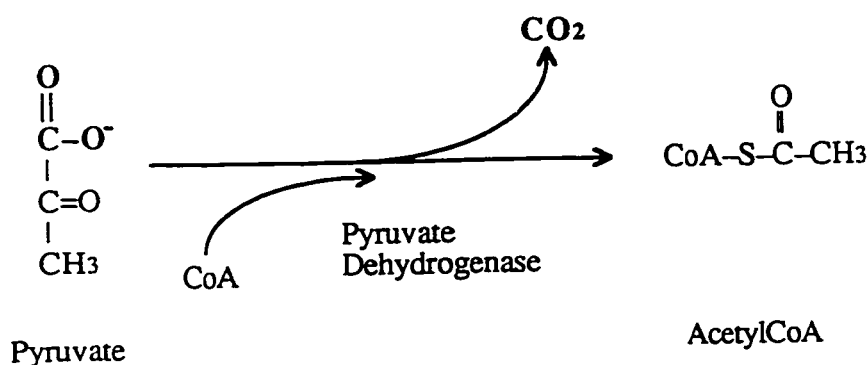


Fig.1.1. Metabolic pathways involved in pyruvate metabolism.

Glucose is degraded glycolytically to pyruvate (PYR), which under aerobic conditions, can either enter the tricarboxylic acid (TCA) cycle via acetylCoA (formed by dehydrogenation, decarboxylation and condensation with coenzyme A) to be metabolized to CO_2 and water, or be condensed with CO_2 to provide net formation of oxaloacetate (OAA). $^{14}\text{CO}_2$ production from $[1-^{14}\text{C}]$ pyruvate specifically indicates formation of acetylCoA, whereas $[2-^{14}\text{C}]$ or $[3-^{14}\text{C}]$ pyruvate leads to formation of $^{14}\text{CO}_2$ in the TCA cycle. (From Hertz and Peng, 1992a).

In addition to being converted to acetylCoA, pyruvate can be condensed with one molecule of CO₂ to provide net formation of oxaloacetate (CO₂ fixation). CO₂ fixation is catalyzed by pyruvate carboxylase, which is present in astrocytes but absent in neurons (Yu et al., 1983; Shank et al., 1985). This reaction does not generate energy, but it leads to net synthesis of a TCA cycle intermediate (Hertz and Peng, 1992a).

In the reaction from pyruvate to acetylCoA (scheme 1), carbon atom no.1 in pyruvate is released as CO₂. Thus, formation rates of labeled CO₂ from [1-¹⁴C]pyruvate provide a direct and under suitable conditions relatively accurate determination of metabolic flux from pyruvate to acetyl CoA.



Scheme 1

1.2.3.2. Properties of pyruvate dehydrogenase

Pyruvate dehydrogenase (PDH) complex, the enzyme that catalyzes the oxidative decarboxylation of pyruvate, is located exclusively in the mitochondrial matrix (Lai et al., 1977; Lai and Clark, 1979). It is a multimolecular aggregate of three enzymes: pyruvate decarboxylase, dihydrolipoyl transacetylase, and dihydrolipoyl dehydrogenase (Lai and Clark, 1989; Reed, 1981). Each catalyzes a part of the overall

reaction. Their physical association links the reactions in proper sequence without the release of intermediates.

The pyruvate dehydrogenase component complex is regulated by a calcium mediated phosphorylation (inactivation) and dephosphorylation (activation) . Ca^{2+} activates PDH by increasing the proportion of nonphosphorylated PDH (PDHa). Ca^{2+} may act as a bridging ligand between pyruvate dehydrogenase phosphatase and the transacetylase subunits that form the core of the PDH complex (Teague et al., 1982).

The changes in PDH activities could play a central role in the regulation of cell metabolism. Pyruvate oxidation in the brain has several unique aspects. Glucose is the most important energy source in the brain, and PDH is one of the key enzymes controlling the catabolism for glycolytic products in mitochondria by allowing increases in mitochondrial ATP synthesis (Denton and McCormack, 1990). Moreover, acetylCoA is derived from the PDH reaction, and acetylcholine metabolism is closely linked to pyruvate oxidation (Hirsch and Gibson, 1982).

1.2.3.3. Role of Ca^{2+} in regulation of pyruvate metabolism

PDH was found to be sensitive to Ca^{2+} within the regulated micromolar concentration range (Denton et al, 1972). The most recent measurements in rat heart mitochondria suggest that PDH is regulated by changes in intramitochondrial Ca^{2+} over the approximate range 0.03-3 μM , with K_a values of around 0.4 - 0.8 μM (McCormack and Denton, 1993). Studies on muscle and heart have shown that the adrenaline-induced increases in PDHa can be correlated to elevated intracellular free calcium (Rutter, et al., 1989). A rise in $[\text{Ca}^{2+}]_i$ is required for K^+ -induced increases in PDHa in isolated nerve terminals (i.e. synaptosomes) (Huang et al., 1994a). If the rise in $[\text{Ca}^{2+}]_i$ is blocked in

putatively calcium free medium, then PDHa does not increase even with K⁺ depolarization. The response of PDH is diminished in EGTA containing medium.

Studies with fura-2-loaded isolated rat heart mitochondria revealed a close relationship between cytosolic and intramitochondrial Ca²⁺. At low extramitochondrial concentrations of Ca²⁺, in the presence of physiological concentrations of Na⁺ and Mg²⁺, the concentration of Ca²⁺ within mitochondria is less than that outside the mitochondria, but there is a preferential transfer of the Ca²⁺ signal from the extramitochondrial medium into the matrix as the concentration of extramitochondrial Ca²⁺ is increased (McCormack et al., 1989).

The Ca²⁺ entry into the mitochondria can be prevented by specific drugs, e.g., ruthenium red (Moore, 1971), which, accordingly, can be used to establish potential correlations between an increase in mitochondrial Ca²⁺ concentration and metabolic events. In heart muscle and certain other tissues, noradrenaline increases pyruvate metabolism by an increase in intramitochondrial Ca²⁺ which, in turn, is secondary to an increase in cytosolic Ca²⁺ and is inhibited by ruthenium red (McCormack and Denton, 1993)

1.2.4. Interaction between neurons and astrocytes

1.2.4.1. Interaction in potassium homeostasis

During neuronal activity, potassium is released to the relatively small extracellular space, leading to an increase in extracellular potassium concentration (Sykova, 1983; Walz and Hertz, 1983). Potassium ions are actively accumulated into astrocytes by (1) exchange with sodium, which is catalyzed by Na⁺, K⁺-ATPase. This

process occurs in both astrocytes and neurons but it is only stimulated by above-normal extracellular potassium levels in astrocytes (Grisar et al., 1979; Huang et al., 1994b); (2) an electroneutral co-transport of K^+ , Na^+ and Cl^- , which is present in astrocytes but not in neurons. Astrocytes also reduce local increases in extracellular K^+ by passive, current-carried redistribution of K^+ through an astrocytic syncytium by the so-called spatial buffering (for discussion, see Hertz 1986; 1990b).

1.2.4.2. Interaction in metabolism

After being released into the synaptic cleft, glutamate is mainly accumulated into astrocytes, where it is partly converted to glutamine by glutamine synthetase which is present only in glial cells (Norenberg and Martinez-Hernandez, 1979). Glutamine is subsequently released to the extracellular space and partly re-accumulated into neurons to synthesize glutamate and GABA. However, much of the accumulated glutamate (how much probably depends upon the experimental conditions) is not converted to glutamine but used as a metabolic fuel by oxidation to CO_2 and H_2O in astrocytes (Hertz and Schousboe, 1986).

As mentioned before, in brain, carboxylation of pyruvate to oxaloacetate only occurs in astrocytes; therefore, net synthesis of α -ketoglutarate (α -KG) from glucose in the TCA cycle can also only take place in these cells. The synthesized TCA cycle intermediates or their metabolites are released and then taken up by neurons (Kaufman and Driscoll, 1992). Extracellular α -KG can be used for net synthesis of glutamate in glutamatergic neurons (Peng et al., 1991).

Astrocytes also synthesize the amino acid alanine in large amounts from pyruvate (Yudkoff et al., 1986), and alanine is reconverted in neurons to pyruvate during

the formation of glutamate from α -KG. The pyruvate thus formed can be used by the neurons as a metabolic substrate (Hertz et al., 1994).

2. HYPOTHESES AND OBJECTIVES

2.1. Hypotheses

Work by McCormack and Denton (1993) has suggested that noradrenaline stimulates pyruvate dehydrogenation in heart muscle via an increase in $[Ca^{2+}]_i$. This finding has led to the hypothesis that neurotransmitters, which increase the cytosolic concentration of calcium, may enhance pyruvate dehydrogenation in astrocytes.

The hypotheses to be tested are:

- 1) Noradrenaline increases $[Ca^{2+}]_i$ and stimulates pyruvate dehydrogenation in primary cultures of astrocytes.
- 2) Dexmedetomidine increases $[Ca^{2+}]_i$ and stimulates pyruvate dehydrogenation in primary cultures of astrocytes.
- 3) Chronic treatment with lithium inhibits the noradrenaline induced increase in $[Ca^{2+}]_i$ in primary cultures of astrocytes.
- 4) Serotonin increases $[Ca^{2+}]_i$ and stimulates pyruvate dehydrogenation in primary cultures of astrocytes.
- 5) Fluoxetine increases $[Ca^{2+}]_i$ and stimulates pyruvate dehydrogenation in primary cultures of astrocytes.
- 6) Chronic treatment with fluoxetine alters the response to acute administration of fluoxetine and serotonin in primary cultures of astrocytes.
- 7) The purinergic transmitters adenosine and guanosine increase $[Ca^{2+}]_i$ in primary cultures of astrocytes.
- 8) The peptidergic transmitter vasopressin increases $[Ca^{2+}]_i$ and stimulates pyruvate dehydrogenation in primary cultures of astrocytes.

2.2. Objectives

The purpose of this study was to investigate the effects of selected adrenergic, serotonergic, purinergic and peptidergic agonists on calcium signalling and pyruvate metabolism in primary cultures of mouse astrocytes in order to establish a possible correlation between these two parameters.

In more detail, the objectives are:

- 1) to determine the effects of the adrenergic agonists noradrenaline, phenylephrine, isoproterenol, clonidine, dexmedetomidine on free intracellular calcium concentration and pyruvate metabolism in astrocytes;
- 2) to determine the effects of the serotonergic agonists serotonin and fluoxetine on $[Ca^{2+}]_i$ and pyruvate dehydrogenation in astrocytes;
- 3) to determine the effects of the purinergic agonists adenosine and guanosine on $[Ca^{2+}]_i$ in cultured astrocytes;
- 4) to determine the effects of the peptide hormone vasopressin on $[Ca^{2+}]$ and pyruvate dehydrogenation in cultured astrocytes.

3. MATERIALS AND METHODS

3.1. Materials

Arginine vasopressin and the V₁ selective antagonist, d(CH₂)₅Tyr(me)-AVP (the Manning Compound) were obtained from Bachem, Inc., Torrance, CA, U.S.A.

CGS 15943 was from CIBA-GEIGY Corporation, Summit, NJ, U.S.A.

Dexmedetomidine was a gift from the Research Center of Farnos-Group Ltd., Orion Co., Turku, Finland.

1,3-Dipropyl-7-methylxanthine (DPMX), ketanserin tartrate and idazoxan HCl were purchased from RBI (Research Biochemicals Incorporated), Natick, MA, U.S.A.

Fluoxetine HCl was donated by Lilly Laboratories, Indianapolis, IN, U.S.A.

Indo-1/acetoxymethyl esters (Indo-1/AM, I-1223, *special packaging*) and Pluronic F-127 were purchased from Molecular Probes, Inc., Eugene, OR, U.S.A.

Lithium chloride was from BDH Inc., Poole, England.

Phenol reagent was from BDH Inc., Toronto, Canada.

[1-¹⁴C] pyruvate (specific radioativity , 11.6-13.5 mCi/mmol) was from Du Pont Canada Inc., Mississauga, ON, Canada.

Scintillation Liquid EcoLite(+)TM was purchased from ICN, Costa Mesa, CA, U.S.A.

All other pharmacological agents and chemicals for preparation of tissue culture medium and experimental solutions were obtained from Sigma Chemical Company, St. Louis, MO, U.S.A.

3.2. Preparation of Mouse Astrocytes in Primary Cultures

The method used for preparing primary cultures of mouse astrocytes was modified by Hertz et al. (1982; 1985a,b; 1989b) and Juurlink and Hertz (1992) from the procedure first described by Booher and Sensenbrenner (1972). Details are as below:

1. Kill newborn (0-24h) Swiss mouse pups by CO₂. Dip the head ends in 70% ethanol and cut off the heads. Place cerebral hemispheres in modified Eagle's Minimum Essential Medium (Table 3.1.) with 20% horse serum (HyClone® Laboratories, Inc., Logan, Utah).
2. Isolate the cerebral hemispheres aseptically from the brains and remove the olfactory bulbs, hippocampal formations, basal ganglia, and meninges, thus isolating the neopallium, i.e., the cortical tissue above and lateral to the lateral ventricles.
3. Cut neopallia into 1 mm cubes and vortex tissue at maximum speed (Duluxe Mixer, Scientific Products, McGaw Park, IL) for 1 min. Pass the cell suspension through Nitex® nylon meshes of 75µm and subsequently 10 µm pore size (L. and S.H. Thompson and Co. Ltd., Montreal, QUE, Canada).
4. Plant cells into 35 mm Falcon Primaria tissue culture dishes (Becton Dickinson Labware, Lincoln Park, NJ), at a density of 20 dishes per brain, corresponding to about 1.5×10^4 cells/cm² and incubate at 37° C in a 95%:5% (v/v) mixture of atmospheric air and CO₂ with a relative humidity of 90%. For [Ca²⁺]_i studies, place two 2.2x1.1 cm diameter glass coverslips (Thomas Scientific® Company) were placed in 35mm Falcon tissue culture dishes and seed the cell suspension onto glass coverslips (10 dishes/brain). Boil the glass coverslips in 2% metso for 20 min and rinse them 6 times with double distilled water before use.
5. After 3 days, replace the medium with fresh medium containing 20% horse serum. From then onwards, feed cultures twice weekly with medium containing 10% horse

serum. After two weeks, add 0.25 mM dibutyryl cyclic adenosine 3',5'-monophosphate (dBcAMP) to the medium.

Such cultures were ready for use between the age of three to six weeks. In these cultures, 95% of the cells were GFAP-positive and they contained no neurons (Hertz et al., 1985a). The main advantage of this preparation is the extensive biochemical and biophysical similarities between these astrocytes grown in vitro and their in vivo counterparts (Hertz et al., 1989b).

Table 3.1. Composition of Modified Eagle's MEM

Chemical	Mol. wt	Amount mg/L	mM
<i>Amino acids</i>			
L-cystine	240.3	48	0.2
L-histidine	155.2	62	0.4
L-isoleucine	131.2	105	0.8
L-leucine	131.2	105	0.8
L-methionine	149.2	30	0.2
L-phenylalanine	165.2	66	0.4
L-threonine	119.1	96	0.8
L-tryptophan	204.2	20	0.1
L-tyrosine	181.2	72	0.4
L-valine	117.2	94	0.8
L-arginine HCl	210.7	253	1.2
L-lysine HCl	182.7	146	0.8
L-glutamine	146.1	365	2.5
<i>Vitamins</i>			
folic acid	--	4.0	--
riboflavin	--	0.4	--
calcium pantothenate	--	4.0	--
choline chloride	--	4.0	--
i-inositol	--	8.0	--
niacinamide	--	4.0	--
pyridoxal HCl	--	4.0	--
thiamine HCl	--	4.0	--
<i>Salts and glucose</i>			
MgSO ₄ ·7H ₂ O	246.5	200	0.8
KCl	74.6	400	5.4
NaHCO ₃	84.0	2200	26.2
NaCl	58.4	6800	116.0
NaH ₂ PO ₄ H ₂ O	138.0	140	1.0
CaCl ₂ ·2 H ₂ O	147.0	264	1.8
D-glucose	180.1	1350	7.5
<i>pH indicator</i>			
phenol red	--	20	--

3.3. Measurement of Intracellular Calcium Concentration

3.3.1. Principle

Indo-1 is a fluorescent dye molecule containing a Ca^{2+} -selective binding site modelled on the chelator, EGTA. The steric arrangement of this binding site gives about $10^5:1$ binding selectivity for Ca^{2+} over the most serious competing ion, Mg^{2+} . This is very important because the free cytosolic $[\text{Mg}^{2+}]$ is about 10^4 times greater than $[\text{Ca}^{2+}]_i$ (Tsien, 1988). Since the hydrophilic portion ($-\text{COO}^-$) of indo-1 prevents the dye molecule from getting into the cytoplasm, an acetoxymethyl ester form of indo-1 (indo-1/AM) is used, which greatly increases the permeability of the dye. After indo-1/AM enters the cell, cytosolic esterases will split off the ester groups and leave the membrane-impermeable part trapped in the cytosol. Upon binding of Ca^{2+} , both of indo-1's emission and excitation spectra shift. Usually indo-1 is excited at 356-365 nm. Upon binding of Ca^{2+} , the emission peak at 405 nm increases while the one at 485 nm decreases (Grynkiewicz et al., 1985) (Figure 3.1.). Since indo-1 shifts emission wavelengths upon binding Ca^{2+} , the ratio, R , of its fluorescence intensities F_{405} and F_{485} (i.e. at wavelengths $\lambda=405$ nm and $\lambda=485$ nm) is sufficient to calculate $[\text{Ca}^{2+}]_i$ (Grynkiewicz et al., 1985). Such a ratio, in principle, cancels out variations in dye loading and local optical path length and compensates for changes in absolute illumination intensity and overall detector sensitivity so long as they are wavelength-independent (Tsien, 1988).

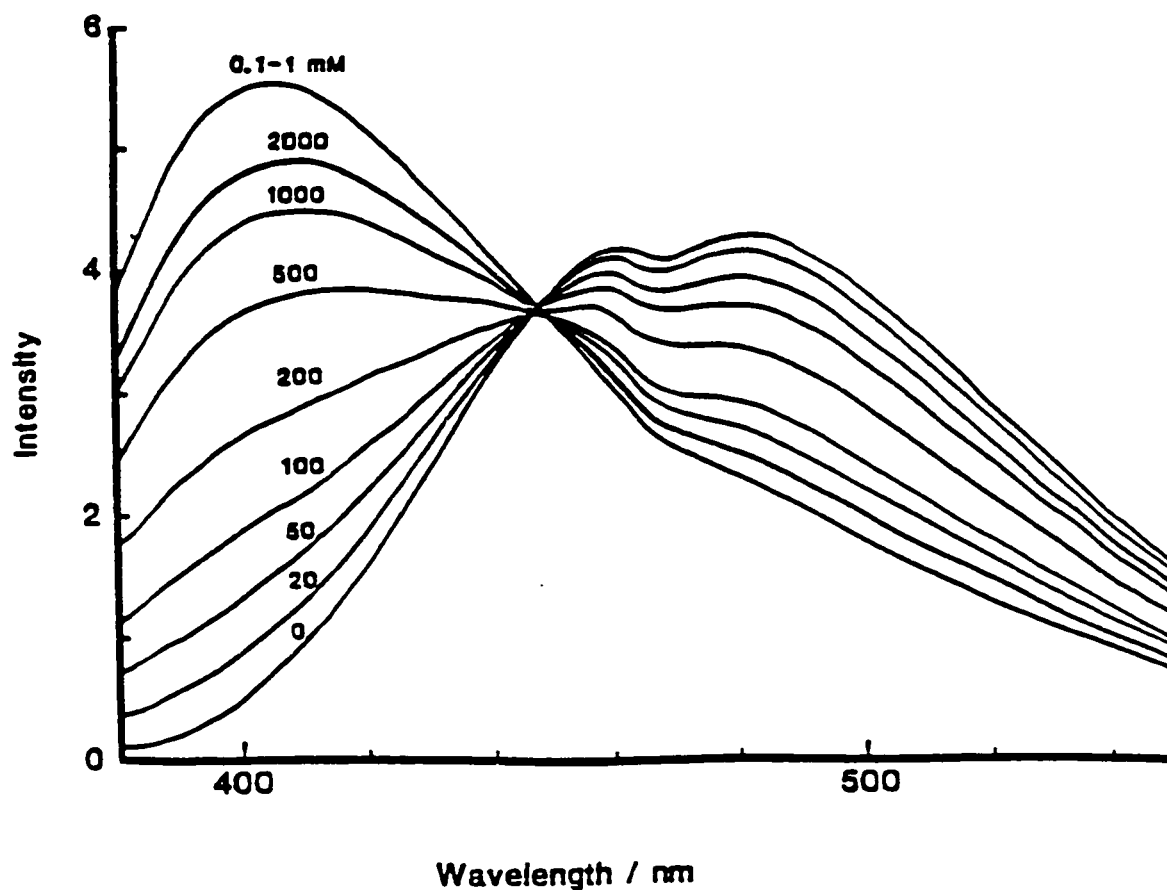


Fig. 3.1. Emission spectra for indo-1 as a function of free Ca^{2+}

The dye, 6 μM , was titrated in 115 mM KCl, 20 mM NaCl, 10 mM MOPS, 1.115 mM MgCl_2 , 1.115 mM $\text{K}_2\text{H}_2\text{EGTA}$, KOH to $\text{pH } 7.050 \pm 0.004$, 37°C , to which small aliquots of K_2CaEGTA were added from a micrometer syringe to raise free Ca^{2+} to the values labeling each curve, in units of nanomolar unless otherwise specified. Excitation was at 355 nm, and both excitation and emission were set to 5 nm band width. Emission spectra show the low-Ca 485 nm peak to be of very similar amplitude to the high-Ca 404 nm peak when excitation is at 355-356 nm (Grynkiewicz et al., 1985).

3.3.2. Instruments for fluorescence measurements

FM-1000 dual-wavelength microfluorimeter (Sycamore Corporation, Santa Barbara, CA, U.S.A.) was used to make the fluorescence measurements. The principal functional components of the FM-1000 system are as follows:

Excitation source: XBO 75W/2 short arc Xenon lamp (OSRAM, Berlin, Germany), dichroic mirror, liquid light guide and 360 nm bandpass filter.

Image/spectral separator (ISS) and detectors: 2 dichroic reflectors, 400nm and 500nm bandpass interference filters, dual high sensitivity photomultiplier tubes with pre-amplifiers.

Video camera: high solution miniature CCD camera (TM-540, PULnix America, Inc., Sunnyvale, CA, U.S.A.) and a video monitor (16", B/W, Panasonic, Osaka, Japan).

Control and detection electronics: dual-channel amplification and filtering for dual-wavelength emission with built-in analog ratio calculation and digital readout of the setting.

A Nikon Diaphot microscope (Nikon Corporation, Tokyo, Japan) was integrated with the FM-1000 microfluorimeter system. A 40x fluor-objective was used to give adequate transmission at wavelengths down to 350 nm. The other instruments used in fluorescence measurement included a flow-through cell chamber (specially ordered from Salt Lake City, UT, U.S. A.) and an EEG & Polygraph Data Recording System (Model 78D, Grass Instruments, Quincy, MA, USA).

3.3.3. Indo-I/AM loading

To dissolve the dye, Pluronic acid F-127 and dimethyl sulfoxide (DMSO) were used. Pluronic F-127 is a nonionic, high molecule weight surfactant polyol. It was used

to help disperse AM ester of Indo-1 and facilitate the dye loading into cells plated on coverslips (Cohen et al., 1974; Poenie et al., 1986).

The dye solution was prepared by the following procedure : 20% (W/W) pluronic F-127 in DMSO was mixed thoroughly in the sonicator; 4 μ l 20% pluronic and 16 μ l of DMSO were added to 50 μ g Indo-1/Am. Since Indo-1 is highly photosensitive, the above steps and the following steps were performed in the dark (only red light, wavelength at 600-700nm). Cultured astrocytes on coverslips were washed once with saline (116 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl_2 , 0.8 mM MgSO_4 , 1.0 mM NaH_2PO_4 , 26.2 mM NaHCO_3 , 7.5 mM glucose) and then loaded with 4 μ M Indo-1 in the incubator at 37° C for 30 min.

3.3.4. Measurement of $[\text{Ca}^{2+}]_i$

For quantitative measurement of $[\text{Ca}^{2+}]_i$, background autofluorescence and photomultiplier tube dark currents must be subtracted from the emission spectrum of the loaded cells. To accomplish this, an unloaded coverslip of cultured cells was placed into the perfusion chamber and the photomultiplier tube outputs offset to zero. This background offset remained stable between experiments. A coverslip of cells loaded with indo-1 was placed in the flow-through cell chamber, which was sitting on the stage of the inverted microscope. A 10 channel buffer perfusion system was attached to the chamber to allow continuous superfusion (1ml/min) of test drugs. Temperature (37°C) regulation was achieved by circulation of warm water with a pump. The image of the

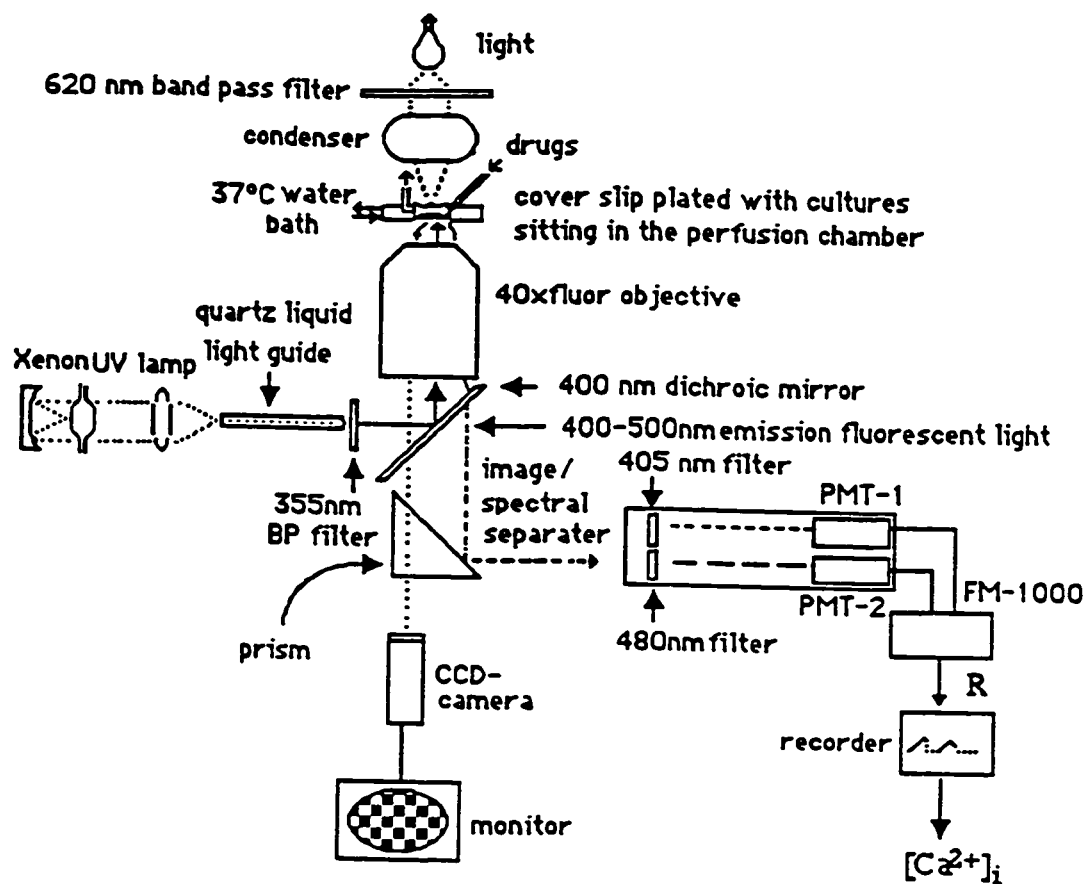


Fig.3.2. Schematic diagram of the experimental set-up

From Zhao (1992).

cells was acquired by illumination via the standard microscope light source passed through a 620 nm bandpass filter. This wavelength was long enough not to interfere with the fluorescence detection at 400 and 500 nm and was suited for the spectral sensitivity of the video camera which was used to display an image of the cells from which a suitable area was selected. After an appropriate area had been chosen on the monitor, the fluorescent dye in the cells was excited by a 355nm UV light from a high pressure 75W xenon short arc lamp, carried by a liquid light guide through a bandpass interference filter. The emitted fluorescent light from the entire 40x objective field was collected by a Fluor 40x objective lens (Nikon, NA 0.85), separated with a dichroic beam splitter and filtered through two bandpass interference filters (400 and 500nm) to permit light of each of the two wavelengths (400 and 500 nm) access to its own, separate photomultiplier tube. The processed dual-wavelength emissions were filtered and amplified by an electronic module. The calculated ratio between the two fluorescent intensities was displayed in a digital readout, and at the same time, the tracing of fluorescent ratio was recorded on the EEG recorder. Fig. 3.2. shows the setup of the $[Ca^{2+}]_i$ measuring device.

3.3.5. Calibration for calculation of Ca^{2+} concentrations

The objective of calibration is to determine the level of fluorescence ratios under conditions of maximum and minimum Ca^{2+} binding. Ca^{2+} measurements using the fluorescent indicators rely on the change in fluorescent properties following Ca^{2+} binding to report the degree of Ca^{2+} saturation of the indicator. At equilibrium, this is related to the free Ca^{2+} concentration, according to the mass action equation:

$$Kd = \frac{C_f \times [Ca^{2+}]}{C_b} \quad \text{giving:} \quad Ca^{2+} = Kd \times \frac{C_b}{C_f} \quad (1)$$

where Kd is the dissociation constant at the fluorescent indicator which binds Ca^{2+} , Cb and Cf are Ca^{2+} -free and Ca^{2+} -bound indicators.

In practice, the equation (1) may be written as:

$$[Ca^{2+}] = Kd \times \frac{(R-R_{min})}{(R_{max}-R)} \times \frac{S_{f2}}{S_{b2}} \quad (2)$$

where R is the fluorescence ratio of Ca^{2+} -bound/ Ca^{2+} -free indicator at rest, R_{min} and R_{max} are the fluorescence ratios at zero Ca^{2+} and saturating Ca^{2+} , and S_{f2}/S_{b2} is the fluorescence ratio for Ca^{2+} -bound/ Ca^{2+} -free indicator measured at the wavelength used to monitor the Ca^{2+} -free indicator (Grynkiewicz et al., 1985).

For the dye Indo-1, the dissociation constant Kd is 250 nM (Grynkiewicz et al., 1985). If Kd , R_{min} , R_{max} , and S_{f2} / S_{b2} are known, the Ca^{2+} concentration can be calculated from equation (2). To obtain the last three parameters, the levels of fluorescence ratios under conditions of maximum and minimum Ca^{2+} binding must be determined. This can be achieved by manipulating intracellular Ca^{2+} . We used digitonin to render the cell permeable to Ca^{2+} so that Ca^{2+} concentrations between the intracellular and the extracellular were rapidly equilibrated. Digitonin selectively permeabilizes the plasma membrane by complexing with cholesterol. Within the range of 0.005-0.10 % (w/v), it permeabilizes the plasma membrane of a wide variety of cells without significantly affecting the gross structure and function of Ca^{2+} -sequestering organelles such as mitochondria and endoplasmic reticulum (Fiskum, 1985).

At the end of most experiments, cells were first exposed to 10 μ M digitonin with PBS containing "zero" Ca^{2+} and 0.5 mM ethyleneglycol-bis-N,N,N',N'-tetraacetic acid (EGTA) until the ratio of 400/500 nm fluorescence declined to a stable plateau

value, and R_{min} and S_{500} were recorded at this moment. After that, the cells were exposed to PBS containing 1.5 mM $CaCl_2$ to obtain R_{max} and S_{500} values.

3.4. Determination of CO_2 production from $[1-^{14}C]$ pyruvate

3.4.1 Principle

As shown in scheme 1, oxidative degradation of pyruvate is initiated by a reaction between coenzyme A and pyruvate, forming acetylCoA by an oxidative decarboxylation. In this reaction, which is catalyzed by the pyruvate dehydrogenase complex, carbon atom no. 1 in pyruvate is quantitatively released as CO_2 . Thus, formation rates of labeled CO_2 from $[1-^{14}C]$ pyruvate provide a direct and probably quite accurate determination of metabolic flux from pyruvate to acetylCoA.

The method used for determination of labeled CO_2 production in cultured astrocytes was described by Yu et al. (1982). $^{14}CO_2$ produced from a labeled substrate is released into the incubation medium and hydrated to carbonic acid. Carbonic acid dissociates to bicarbonate and hydrogen ions. By addition of excess acid, bicarbonate is converted via carbonic acid to CO_2 which can be trapped into an alkaline hydroxide solution in a closed chamber.

3.4.2. Procedure

1. Wash the culture 2 times with PBS.
2. Add 1 ml PBS to each dish; preincubate culture in a 37° C incubator for 10 min.

3. Add 10 μ l of 500 mM pyruvate (5 mM) to each dish, and 0.25 μ Ci of [$1\text{-}^{14}\text{C}$] pyruvate and chemicals in question to each dish.
4. Place the cultures onto glass plates and position a funnel upside down over each culture, sealing the funnel to the glass plate with vacuum grease.
5. Close the funnel with a rubber serum stopper.
6. Put the chamber into an incubator at 37° C for 30 min.
7. At the end of reaction time, inject 0.5 ml of 0.4 M acetic acid into the dish in order to acidify the medium and 2 ml of 1 M NaOH solution into a plastic beaker presuspended in the chamber for trapping of CO₂.
8. Transfer the chamber onto the surface of a 60°C water bath to create air circulation to trap the CO₂ quantitatively into the NaOH.
9. After 30 min, open the chamber and transfer the plastic beaker and 0.1 ml of incubation medium in duplicate separately into scintillation vials containing 10 ml of scintillation fluid.
10. Determine the radioactivity in the NaOH and incubation medium with a LKB RackBeta liquid Scintillation Spectrometer.
11. Add 200 μ l of concentrated perchloric acid to precipitate all protein in the medium, and leave the culture overnight.
12. Scrape the culture and centrifuge the suspension at 1200 g for 5 min.
13. Discard the supernatant and dissolve the pellet in 1 ml of 1 N NaOH and quantitate the protein by the conventional Lowry method.
14. Calculate the rate of CO₂ production from the radioactivity in the NaOH, the specific activity of pyruvate in the medium and the protein content in the culture.

3.4.3. Calculation of CO₂ production

1. Calculate specific activity of labeled compound ([$1\text{-}^{14}\text{C}$] pyruvate):

1) Average medium contents (dpm) in 0.1 ml

$$\text{e.g. } (33913+35738) - \text{blank (28)} = 69623 \div 2 = 34811$$

$$\text{i.e. } 348110 \text{ dpm/ ml}$$

2) Unlabeled compound used:

$$5 \text{ mM pyruvate} = 5 \text{ mmol/L} = 5 \mu\text{mol/ml} = 5000 \text{ nmol/ml}$$

3) Specific activity of pyruvate used:

$$= \text{dpm/ml} \div \text{unlabeled compound concentration}$$

$$= 348110 \text{ dpm/ml} \div 5000 \text{ nmol/ ml}$$

$$= 348110 \text{ dpm} \div 5000 \text{ nmol}$$

$$= 69.62 \text{ dpm / nmol}$$

2. Calculate the CO₂ production during the incubation period using sp. act.:

(flux in this case = ¹⁴CO₂ production)

$$\text{e.g. } 2066 \text{ dpm} - \text{blank} = 2038$$

$$\text{flux} = 2038 \text{ dpm} \div \text{sp.act.} = 2038 \text{ dpm} \div 69.62 \text{ dpm/nmol} = 29.06 \text{ nmol}$$

of CO₂ per sample

3. Calculate actual flux:

Actual flux = (measured production) \div (length of incubation period x total protein/ dish)

$$= 29.06 \div 30 \times 0.147$$

$$= 6.58 \text{ nmol } ^{14}\text{CO}_2 \text{ produced per min, per mg protein}$$

3.5. Determination of Protein

3.5.1. Principle

The protein contents of cultures were measured by the Lowry assay (Lowry et al., 1951). The principle behind the Lowry assay is that an intense blue color develops when substances containing two or more peptide bonds react with alkaline copper sulfate and Folin-Ciocalteu reagent. The colored product is the result of the coordination of peptide nitrogen atoms with Cu^{2+} and the reduction of the Folin-Ciocalteu reagent (phosphomolybdate-phosphotungstate) by tyrosine and tryptophan residues in the protein. The amount of product formed depends on the concentration of protein. In practice, a standard curve is prepared with bovine serum albumin.

3.5.2. Solutions

- A. 2% Na_2CO_3 in 0.1 N NaOH;
- B. 0.5% CuSO_4 ;
- C. 1% Na-K-Tartrate;
- D. 50 ml A + 0.5 ml B + 0.5 ml C;
- E. Phenol reagent 1:1 diluted with distilled water;
- F. Standard solution: 0.0050 g of bovine serum albumin (BSA, Sigma No. A4378) in 10 ml of 0.125M NaOH.

3.5.3. Procedure

1. Dissolve cells in culture dish in 1 ml of 1 M NaOH. Remove 50 μ l of cell solution in duplicate. Add 150 μ l distilled water to each
2. Prepare five standards in duplicate and a blank in triplicate as Table 3.2.

Table 3.2.

Amount. of BSA (μ g/0.2 ml of 0.25 M NaOH)	Standard solution (μ l)	0.125 M NaOH (μ l)
0	0	200
10	20	180
20	40	160
30	60	140
40	80	120
50	100	100

3. Add 1 ml of solution D to all samples and standards and let stand at room temperature for 20 min.
4. Add 0.1 ml of solution E and vortex immediately.
5. After exactly 30 min at room temperature, read the absorbance against the blank at 500 nm with spectrophotometer (Spectronic® 2000, Bausch & Lomb, Rochester, NY).
6. Calculate the content of protein in each culture based on the standard curve.

3.6. Statistical Analysis

Statistical analysis was carried out using computer software package "StatView SE+Graphics™", version 1.03 (Abacus Concepts, Inc. Berkeley, CA, USA). Values are

reported as mean \pm S.E.M. One-way analysis of variance (ANOVA) followed by Fisher PLSD's test was applied for multiple comparisons between groups; Student's t-test was used to detect significant differences between two groups. The null hypothesis was rejected at the 95% level, considering a P value < 0.05 as significant.

4. RESULTS

4.1. Effects of adrenergic agonists on free cytosolic calcium and pyruvate dehydrogenation in cultured astrocytes

4.1.1. Calcium response evoked by adrenergic agonists

Figure 4.1. shows a fluorescence recording from primary cultures of astrocytes using Indo-1/AM, in which the cells were superfused continuously with a salt buffer to yield a steady baseline in the resting $[Ca^{2+}]_i$. Calibration to determine the absolute calcium concentration was done at the end of the experiment. When noradrenaline was added, the intracellular calcium concentration was found to undergo changes with subsequent return to the baseline after removal of the drug.

To strengthen the view that the noradrenaline-induced increase in $[Ca^{2+}]_i$ is mediated by receptors, the effect of two antagonists, phentolamine (100 μ M) and yohimbine (100 μ M) were examined. The astrocyte cultures first were perfused 1 min with a salt solution containing phentolamine or yohimbine, followed by one containing an antagonist plus noradrenaline (1 μ M). The response to noradrenaline was abolished by both phentolamine and yohimbine (Fig. 4.2. and 4.3.). Addition of 1 μ M of noradrenaline onto the same culture before and after the antagonist had been administered (with a wash time of at least 3 min), caused a virtually identical response. This indicates 1) that no irreversible alteration has been evoked by the antagonist and 2) that there is no desensitization of the noradrenaline response. Accordingly different concentrations of noradrenaline can be tested in the same culture.

Figure 4.4. illustrates the effects of different concentrations of noradrenaline, a mainly α adrenergic agonist, on $[Ca^{2+}]_i$. The average resting concentration of calcium ranged between 50 and 150 nM. At 10 μ M, noradrenaline $[Ca^{2+}]_i$ increased to about 600 nM. It can be seen that a half-maximum increase in $[Ca^{2+}]_i$ required a noradrenaline concentration of between 1-3 μ M.

Phenylephrine, a selective agonist of α_1 -adrenergic receptors, induced an increase in $[Ca^{2+}]_i$ (Fig. 4.5). Ten μ M of phenylephrine caused an increase of $[Ca^{2+}]_i$ from a resting level of 100 nM to about 600 nM. The phenylephrine-evoked response was completely abolished by the simultaneous administration of phentolamine (100 μ M) (Fig. 4.6.).

The dose-response curve presented in figure 4.7. shows that clonidine increased $[Ca^{2+}]_i$ significantly. The stimulation reached a maximum at a concentration of 1 μ M. The response evoked by clonidine could be entirely blocked by yohimbine, as shown in Figure 4.8.

An increase in $[Ca^{2+}]_i$ was seen at 50 nM dexmedetomidine, but a significantly smaller effect was observed at 100 nM. During exposure to still higher concentrations of dexmedetomidine there was a marked rise. Thus the response was biphasic. The whole concentration-dependence relation of the elevation in $[Ca^{2+}]_i$ caused by dexmedetomidine is shown in Figure 4.9. Both yohimbine and idazoxan inhibited the increase in $[Ca^{2+}]_i$ evoked by 25 μ M dexmedetomidine although neither drug completely abolished the effect (Fig.4.10.)

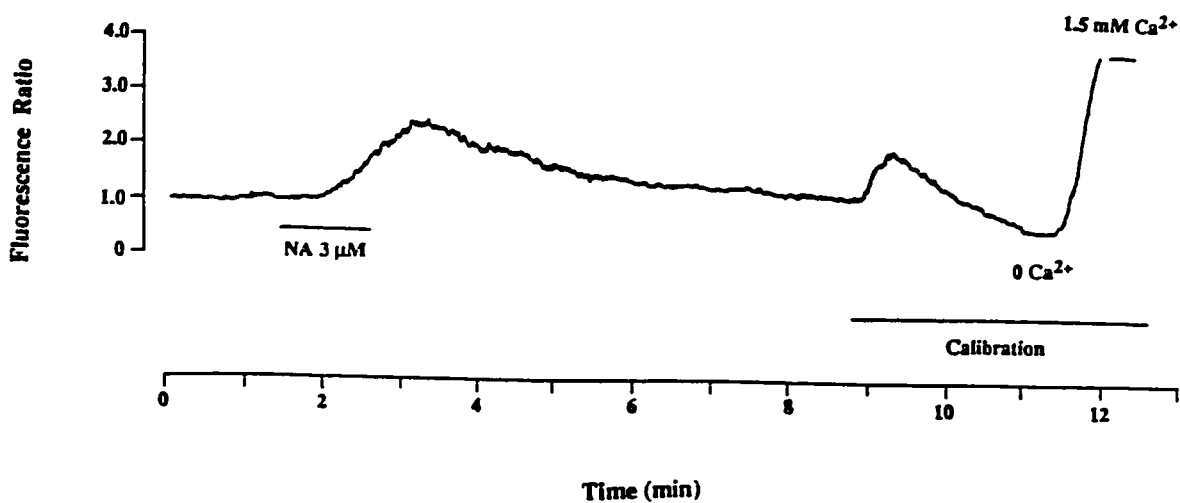


Fig. 4.1. Tracing of free cytosolic calcium concentration in a culture of mouse astrocytes

A representative recording showing the stable baseline, indicating a constant "resting" level of $[\text{Ca}^{2+}]_i$; increase in $[\text{Ca}^{2+}]_i$ upon addition of the adrenergic agonist noradrenaline (NA). Calibration as described in "Methods" was done at the end of the experiment.

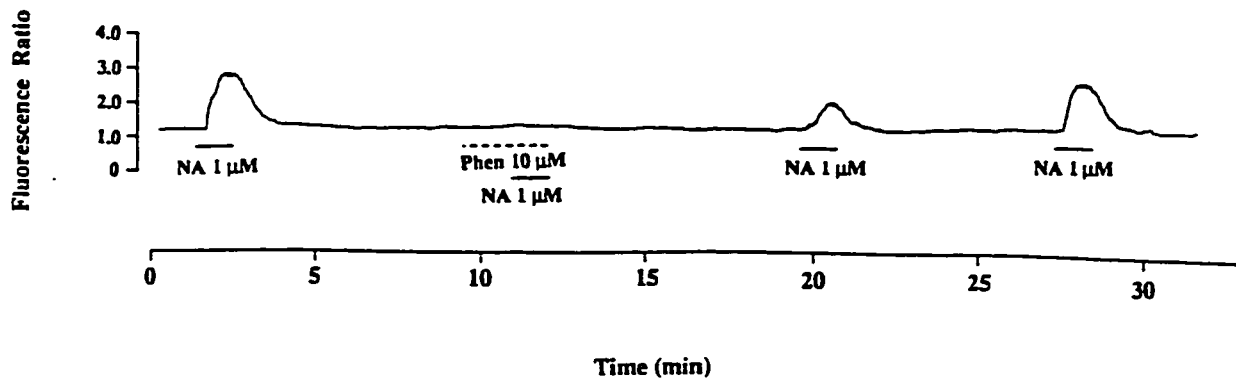


Fig. 4.2. Inhibitory effect of phentolamine on noradrenaline-stimulated $[Ca^{2+}]_i$ increase in astrocytes

Cells were perfused with 1 μ M of noradrenaline for 1 min before and after the application of phentolamine. Phentolamine 10 μ M (Phen) was added into cells for 1 min followed by phentolamine plus noradrenaline 1 μ M. Cells were superfused and allowed recovery time between each drug application. Similar results were obtained in a second experiment.

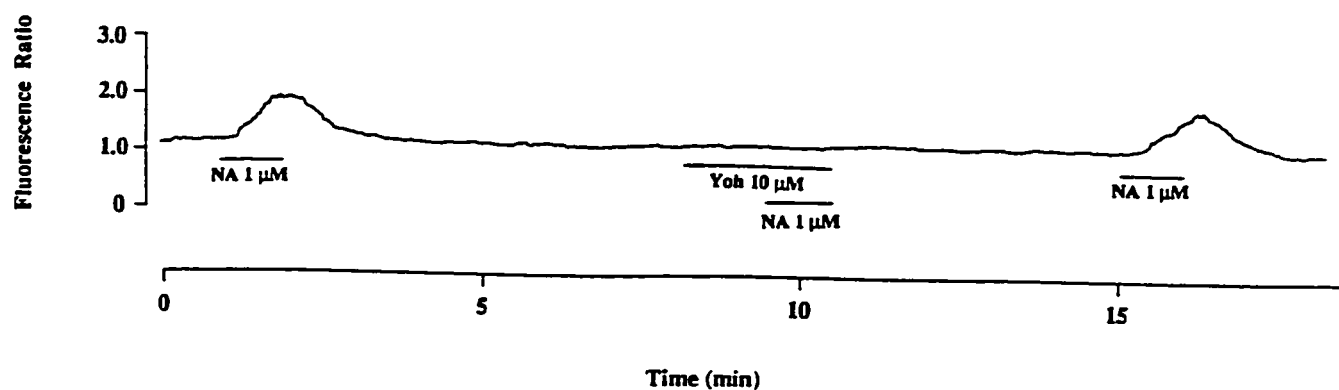


Fig. 4.3. Inhibitory effect of yohimbine on noradrenaline-stimulated $[Ca^{2+}]_i$ increase in astrocytes

Cells were perfused with 1 μ M of noradrenaline for 1 min before and after the application of yohimbine. 10 μ M yohimbine (Yoh) was added into cells for 1 min followed by additional yohimbine plus noradrenaline 1 μ M. Cells were superfused and allowed recovery time between each drug application. Similar results were obtained from 3 more experiments of 2 different batches of cultures.

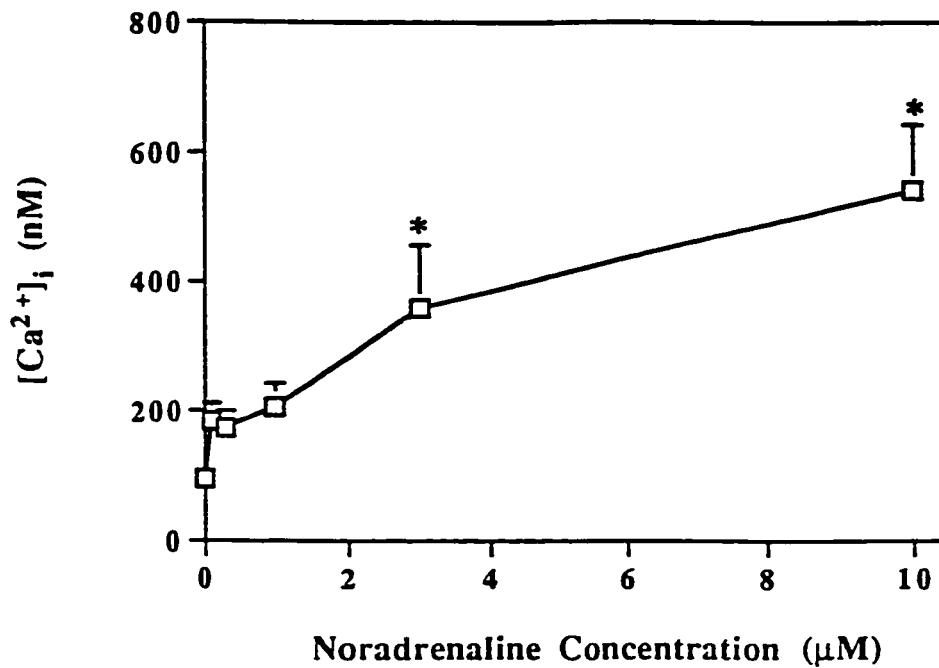


Fig. 4.4. Concentration-response relationship for noradrenaline-evoked changes in $[Ca^{2+}]_i$ in astrocytes

Cells were exposed to various concentrations of noradrenaline for 1 min. Noradrenaline-evoked Ca^{2+} responses were detected at $0.1\mu M$, and the increase in $[Ca^{2+}]_i$ was significantly different from $3\mu M$ (*) and onwards. Each value shown represents the mean \pm S.E.M. of 5-9 individual experiments from 3 different batches of cultures.

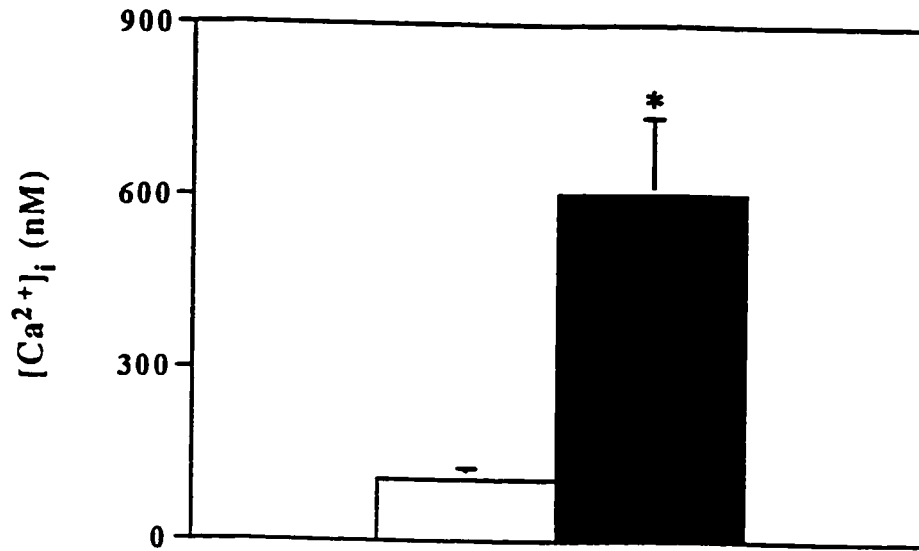


Fig. 4.5. Effect of phenylephrine on $[Ca^{2+}]_i$ in astrocytes

10 μ M of phenylephrine caused an increase in $[Ca^{2+}]_i$ from 100 nM of control (□) to 600 nM (■). Columns represent the means \pm S.E.M. of 4 individual experiments, using cultures from 2 different batches. * denotes $P < 0.05$ vs. control value.

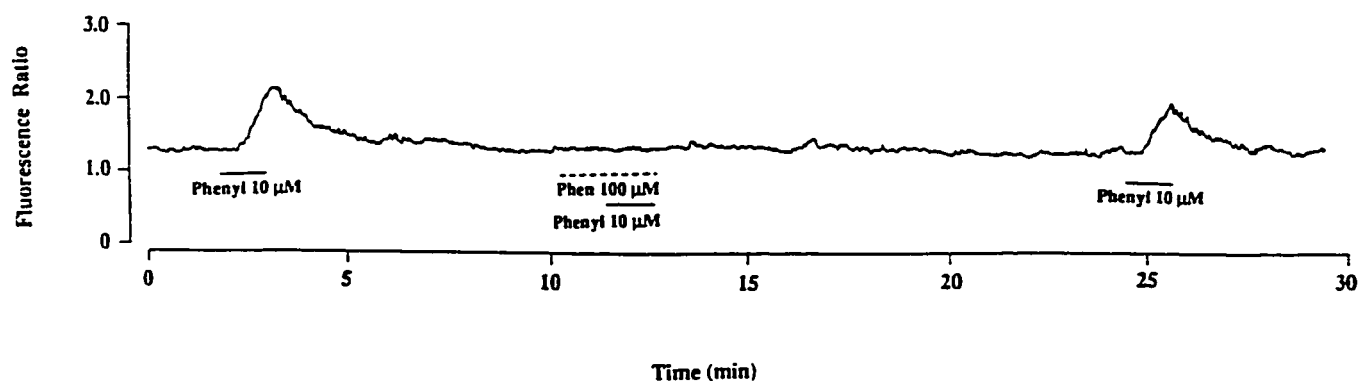


Fig. 4.6. Effect of phentolamine on phenylephrine-evoked $[Ca^{2+}]_i$ increase in astrocytes

A representative trace showing phenylephrine-induced changes in $[Ca^{2+}]_i$ in the absence and presence of phentolamine. Cells were initially perfused with 10 μ M phenylephrine (Phenyl) to establish a control response, then exposed to 100 μ M phentolamine (Phen) for 1 minute before a second addition of 10 μ M phenylephrine and finally re-exposed to phenylephrine alone. Similar results were seen in a second experiment.

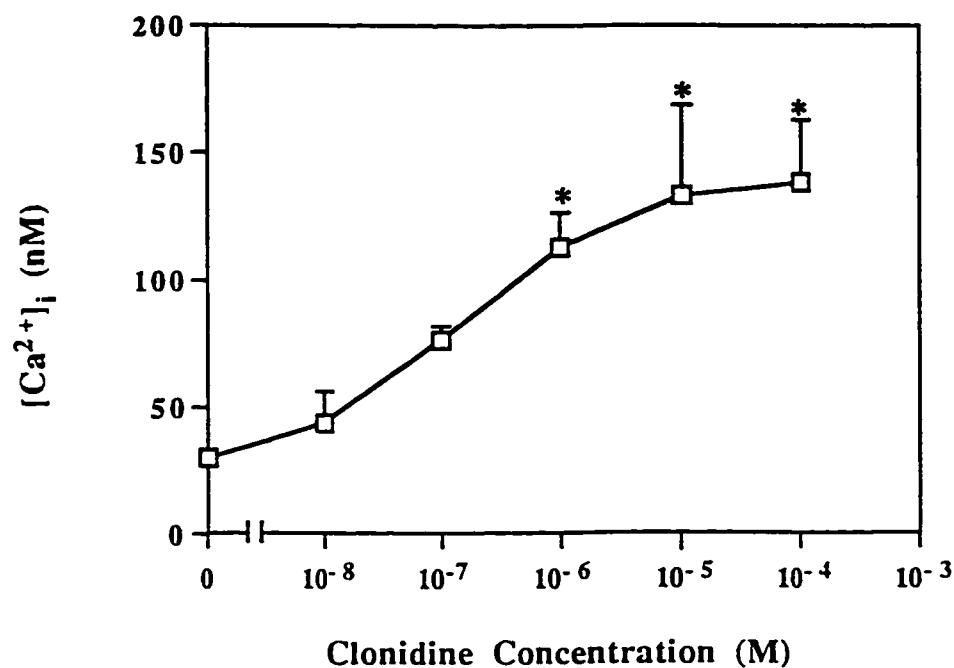


Fig. 4.7. Concentration-response relationship for clonidine-stimulated changes in $[Ca^{2+}]_i$ in astrocytes

Cells were exposed to various concentrations of clonidine for 1 min. The increase in $[Ca^{2+}]_i$ was significantly different from $1\mu M$ (*) and onwards. Each value shown represents means \pm S.E.M. of 3-5 individual experiments, using cultures from 3 different batches.

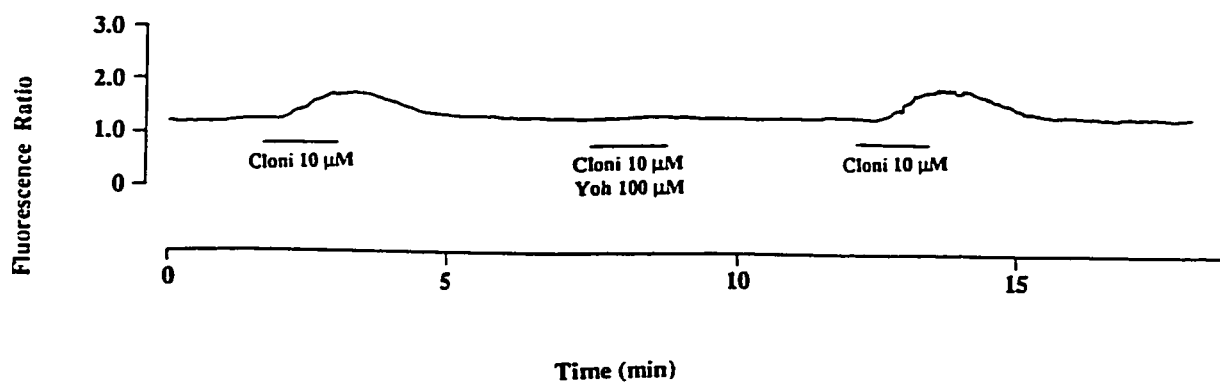


Fig. 4.8. Effect of yohimbine on clonidine-induced $[Ca^{2+}]_i$ increase in astrocytes

A representative trace showing clonidine-induced changes in $[Ca^{2+}]_i$ in the absence and presence of yohimbine. Cells were initially perfused with 10 μ M clonidine (Cloni) to establish a control response, then exposed to 100 μ M yohimbine (Yoh) for 1 minute before a second addition of 10 μ M clonidine and finally re-exposed to clonidine alone. Similar results were seen in 3 more experiments.

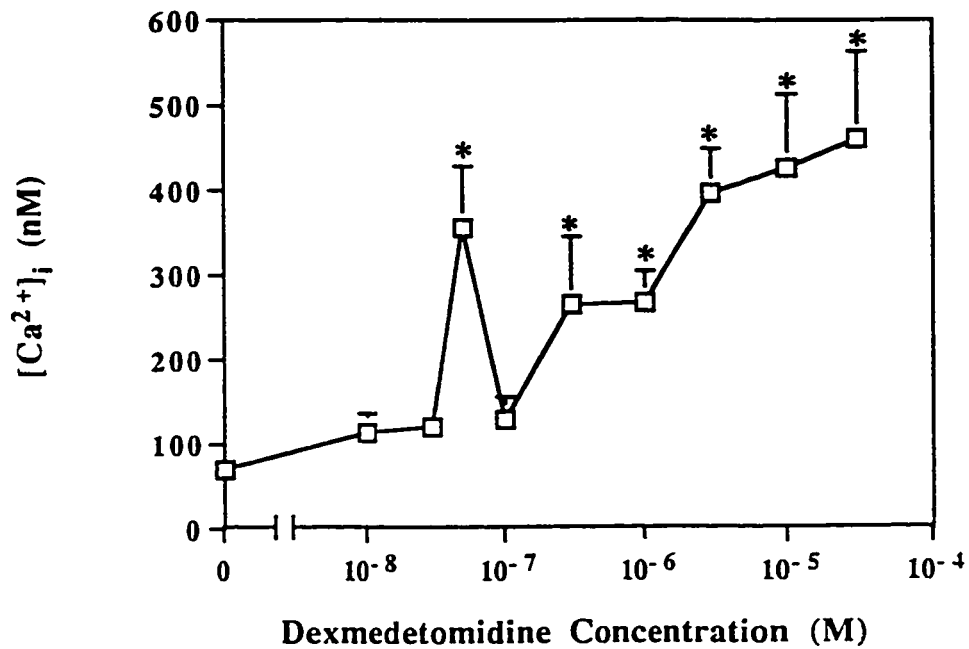


Fig. 4.9. Concentration response relationship of dexmedetomidine effect on [Ca²⁺]_i

A dose-response curve showing the effect of varying concentrations of dexmedetomidine on [Ca²⁺]_i in cultured astrocytes. Results shown are averages \pm S.E.M. of 4-18 individual experiments, using cultures from at least 2 different batches. * indicates the significant difference ($P < 0.05$) from the control.

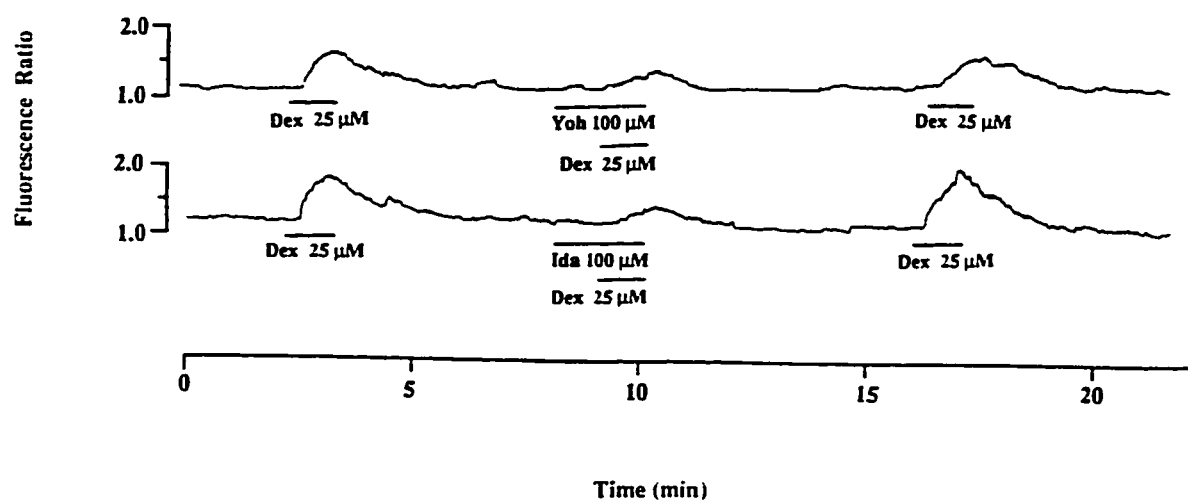


Fig. 4.10. Effect of yohimbine and idazoxan on dexmedetomidine-stimulated $[Ca^{2+}]_i$ increase in astrocytes

Cells were initially perfused with 25 μ M dexmedetomidine to establish a control response, then exposed to 100 μ M yohimbine (upper tracing) or 100 μ M idazoxan (lower tracing) for 1 minute before a second addition of 10 μ M dexmedetomidine and finally re-exposed to dexmedetomidine alone. Similar results were seen from 1 more experiment.

4.1.2. Stimulation of CO₂ production from [1-¹⁴C]pyruvate by adrenergic agonists

The rate of ¹⁴CO₂ production from [1-¹⁴C] pyruvate in primary cultures of astrocytes was 6.19 ± 0.68 (S.E.M) nmol/min/mg protein (number of experiments (n)=67). The effects of different concentrations of noradrenaline on pyruvate dehydrogenation are shown in Fig. 4. 11. It can be seen that noradrenaline significantly stimulated the CO₂ formation by about 50% at a concentration of 0.3 μ M, a steady level of 60-70% was reached at 1 μ M and the noradrenaline concentration giving half maximum stimulation was 0.1 to 0.2 μ M.

In order to establish which receptor subtypes are involved in the stimulation of oxidative metabolism by noradrenaline, the effect of specific adrenergic agonists and antagonists on CO₂ formation from [1-¹⁴C] pyruvate was investigated. Ten μ M noradrenaline (mainly α agonist) and clonidine (α_2 agonist) both stimulated ¹⁴CO₂ production from [1-¹⁴C] pyruvate significantly ($p < 0.05$), whereas isoproterenol showed no effect (Fig. 4.12.) and a tendency towards a small stimulation by phenylephrine (α_1 agonist) was not significant. These findings were mirrored by the effect of noradrenaline plus a subtype specific antagonist (Fig. 4.13.). The α_2 antagonist yohimbine inhibited the noradrenaline-induced CO₂ production to approximately 40% ($p < 0.05$), and the β antagonist propranolol had absolutely no effect. The α_1 agonist prazosin had no significant effect on noradrenaline-induced CO₂ production, but there was a tendency towards an inhibition. The conclusion of both the agonist and antagonist studies is that there is a clear α_2 -adrenergic effect and a possible α_1 -adrenergic effect. If both subtypes are active, it can not be concluded whether they act on the same or on different individual cells.

Since noradrenaline stimulated pyruvate dehydrogenation and increased $[Ca^{2+}]_i$, the effect of calcium depletion on $^{14}CO_2$ production from labeled pyruvate was studied. Cultures were pre-incubated for 1 hour with "zero" calcium PBS containing 7.5 mM glucose and 2.5 mM EGTA PH 7.35; then the medium was changed to PBS with 5 mM pyruvate and 2.5 mM EGTA, without calcium and glucose, before the reaction was started. Table 4. 1. shows that noradrenaline, in the absence of calcium in the medium and the simultaneous presence of the calcium chelating agent EGTA, has no significant effect. A similar result was obtained by deletion of calcium from the incubation medium with an increase of the magnesium concentration (10 mM) (Table 4.2.). However, the stimulation of CO_2 production by noradrenaline was not inhibited by ruthenium red, a blocker of mitochondrial Ca^{2+} uptake (Fig.4.14.). In contrast to noradrenaline, an increase in the concentration of potassium (K^+) in the medium above the control level of 5 mM did not significantly increase pyruvate dehydrogenation (Fig. 4.15.).

Dexmedetomidine also evokes a biphasic increase in pyruvate dehydrogenation. There was a peak effect at 70-100 nM, followed by a statistically significant reduction of the stimulation (to zero percent at 300 nM) and a second, larger peak at 10 μ M (Fig. 4.16.). Yohimbine completely inhibited the stimulation of pyruvate dehydrogenation by dexmedetomidine (Fig. 4.17.). Idazoxan appeared to cause an incomplete inhibition, but the remaining stimulation by dexmedetomidine in the presence of idazoxan was not statistically significant.

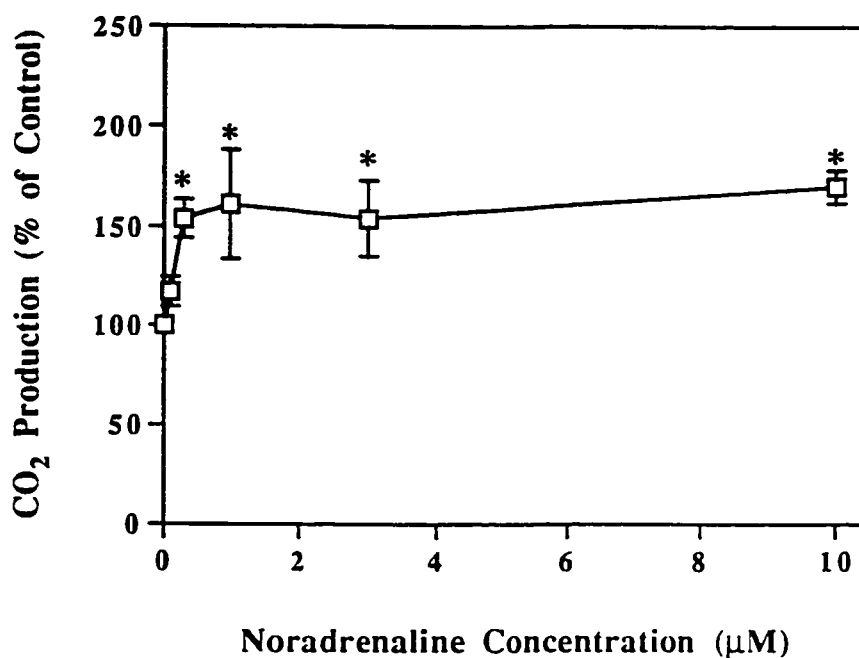


Fig. 4.11. Concentration response relationship of noradrenaline effect on $^{14}\text{CO}_2$ production from $[1-^{14}\text{C}]$ pyruvate in primary cultures of astrocytes

Cells were incubated with various concentrations of noradrenaline for 30 min. Results are means \pm S.E.M. of 6 (1.3 μM), 9 (0.1 μM), 14 (0.3 μM) and 20 (control) individual experiments, using cultures from at least 2 different batches. * These values are significantly different ($p < 0.05$) from control condition.

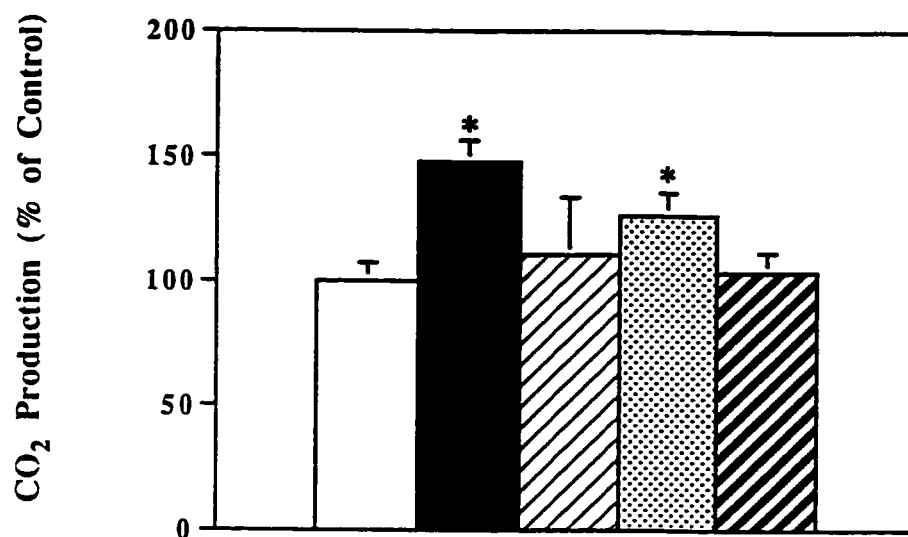


Fig. 4.12. Effects of adrenergic agonists on CO₂ production from [1-¹⁴C]pyruvate

Cultures were incubated with 10 μ M of either noradrenaline (■), phenylephrine (▨), clonidine (▤), or isoproterenol (▩) for 30 min and labelled CO₂ was measured. Values are expressed as % of control (□, CO₂ formation in the absence of any added adrenergic agonists). Each value represents the average \pm S.E.M. of 6-15 individual experiments from at least two batches of cultures. * These values are significantly different from the control value (P < 0.05).

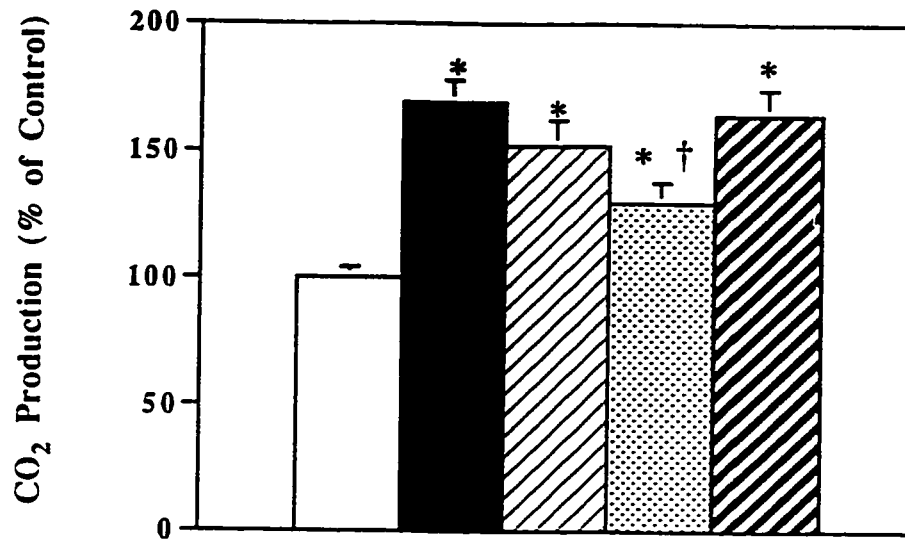


Fig. 4.13. Effect of adrenergic antagonists on noradrenaline -stimulated CO₂ formation from [1-¹⁴C] pyruvate

Cultures were incubated for 30 min with 10 μ M of noradrenaline alone (■) or in the presence of 100 μ M adrenergic antagonist prazosin (▤), yohimbine (▦), and propranolol (▧). Values are expressed as % of control (□, CO₂ formation in the absence of any adrenergic agonist). Each value represents the average \pm S.E.M. of 7-14 individual experiments from at least 2 batches of cultures. * These values are significantly different from the control. † This value is significantly different from the value obtained in the presence of noradrenaline alone (P < 0.05).

Table 4.1. Effect of noradrenaline (0.3 μ M) on rate of $^{14}\text{CO}_2$ production from [1- ^{14}C]pyruvate under control condition and in a Ca^{2+} free medium containing 2.5 mM EGTA.

	Pyruvate oxidation (nmol/min / mg protein)	
	Control medium	No Ca^{2+} , 2.5 mM EGTA
Control	4.68 \pm 0.85	2.58 \pm 0.26
Noradrenaline	8.21 \pm 1.12*	3.20 \pm 0.16**

Results are means \pm S.E.M. of 7 individual experiments, using 2 different batches of cultures.

* significantly different from the control value ($p < 0.05$).

Table 4.2. Effect of noradrenaline (0.3 μ M) on rate of $^{14}\text{CO}_2$ production from [1- ^{14}C] pyruvate under control condition and in a high Mg^{2+} /no Ca^{2+} medium.

	Pyruvate oxidation (nmol/min / mg protein)	
	Control medium	No Ca^{2+} , 10 mM Mg^{2+}
Control	2.65+0.31	2.55+0.28
Noradrenaline	3.77+0.41*	2.35+0.19

Results are means + S.E.M. of 9 individual experiments from 3 different batches of astrocytes.

* significantly different from the control value ($p < 0.05$).

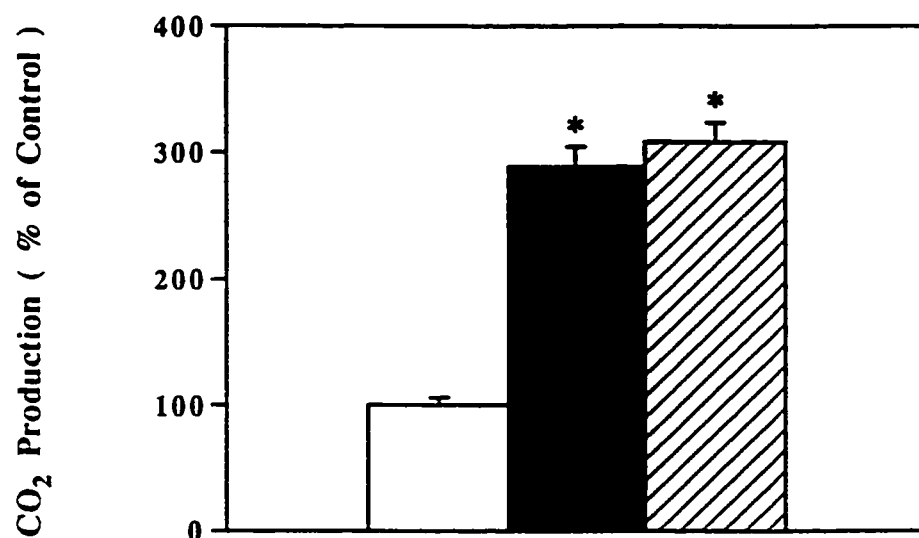


Fig. 4.14. Effect of ruthenium red on CO₂ production stimulated by noradrenaline

100 μM ruthenium red was included in the incubation medium for 45 minutes before noradrenaline was added. Experimental conditions are control (□); 10 μM noradrenaline (■); and 10 μM noradrenaline plus 100 μM ruthenium red (▨). Results are means ± S.E.M. of 3-6 individual determinations.

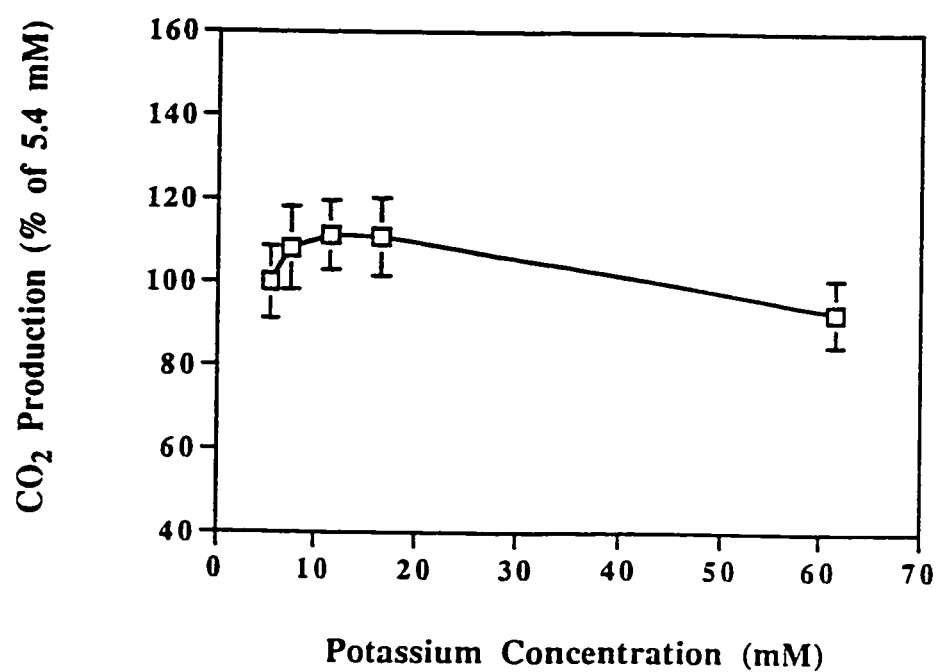


Fig. 4.15. Effect of elevated extracellular potassium concentration on CO₂ production from [1-¹⁴C] pyruvate

Cells were incubated in various concentrations of potassium for 30 min. Results are the averages \pm S.E.M. of 9-12 individual experiments from at least 2 batches of cultures.

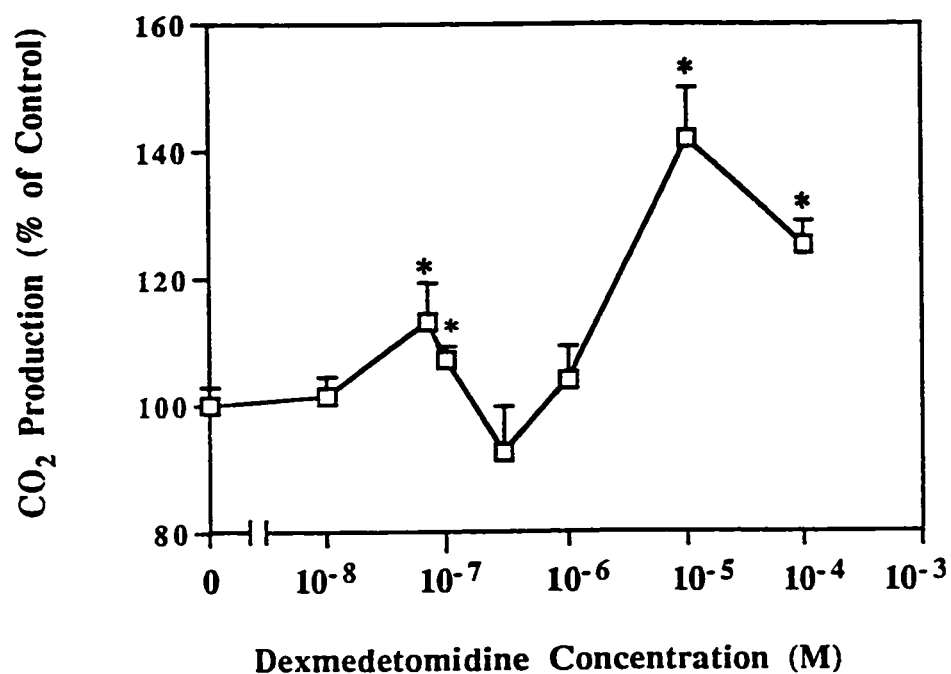


Fig. 4.16. Concentration response curve for effect of dexmedetomidine on CO₂ production from [1-¹⁴C] pyruvate

Cells were incubated with various concentrations of dexmedetomidine for 30 min. Each value represents the average of 4-10 individual experiments from 2 batches of cultures. * indicates the value is significantly different from the absence of dexmedetomidine ($P < 0.05$).

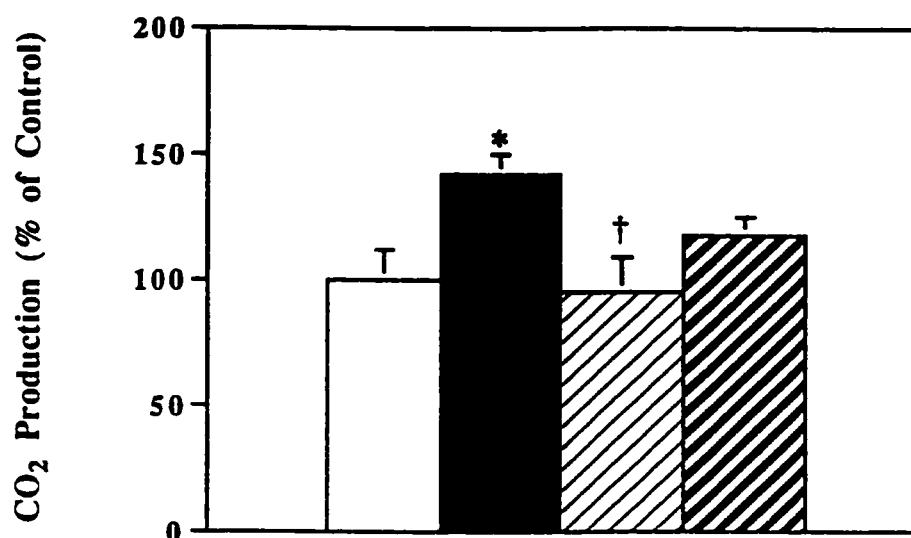


Fig. 4.17. Inhibitory effect of α_2 -adrenergic antagonists on dexmedetomidine-induced CO₂ production

Cultures were incubated without dexmedetomidine (□), 10 μ M dexmedetomidine (■), 10 μ M dexmedetomidine combined with 100 μ M yohimbine (▨), and 10 μ M dexmedetomidine combined with 100 μ M idazoxan (▩). Values are means \pm S.E.M. of 6 individual experiments from 2 batches of cultures.

* This value is significantly different from the corresponding control value ($P < 0.05$).

† This value is significantly different from the corresponding value in the presence of dexmedetomidine alone ($P < 0.05$).

4.1.3. Inhibition of noradrenaline stimulated increase in $[Ca^{2+}]_i$ in astrocytes by chronic treatment with lithium

In this study, lithium chloride concentration of 1 mM was added to the culture medium for 7-14 days. Other cultures from the same batch were left untreated as drug-naive controls and studied in parallel with the drug-treated cultures. In studies of acute effects of lithium, 1 mM lithium chloride was present from the beginning of the dye loading and onwards. The chloride salt of lithium was chosen in spite of its hygroscopic properties, and the concomitant increase in chloride concentration was insignificant (<1%) and had no effect on pH. Care was taken to prevent significant uptake of water into lithium chloride during and before weighing by performing all procedures as rapidly as possible.

Fig. 4.18. shows tracings of $[Ca^{2+}]_i$ during a 15-20 min period in two different cultures of astrocytes from the same batch, studied on the same day. Exposure to 1 μ M noradrenaline caused an increase in $[Ca^{2+}]_i$ which is larger in the upper tracing, obtained from an untreated culture than in the lower tracing, representing a culture which had been treated for 14 days with 1 mM lithium.

The values for basal $[Ca^{2+}]_i$ were 50-100 nM in untreated control cultures but it was significantly reduced in cultures that had been treated with lithium for 7-14 days (Fig. 4.19.). During exposure to 1 μ M noradrenaline, $[Ca^{2+}]_i$ rose to between 550 and 830 nM in untreated control cultures, but significantly less in sister cultures (350 to 500 nM) that had been chronically treated with lithium.

Acute treatment with lithium (during a 30 min period) did not inhibit the noradrenaline induced surge in $[Ca^{2+}]_i$, which rose to the same extent in cultures that were acutely treated with lithium and in untreated sister cultures (Fig. 4.20.).

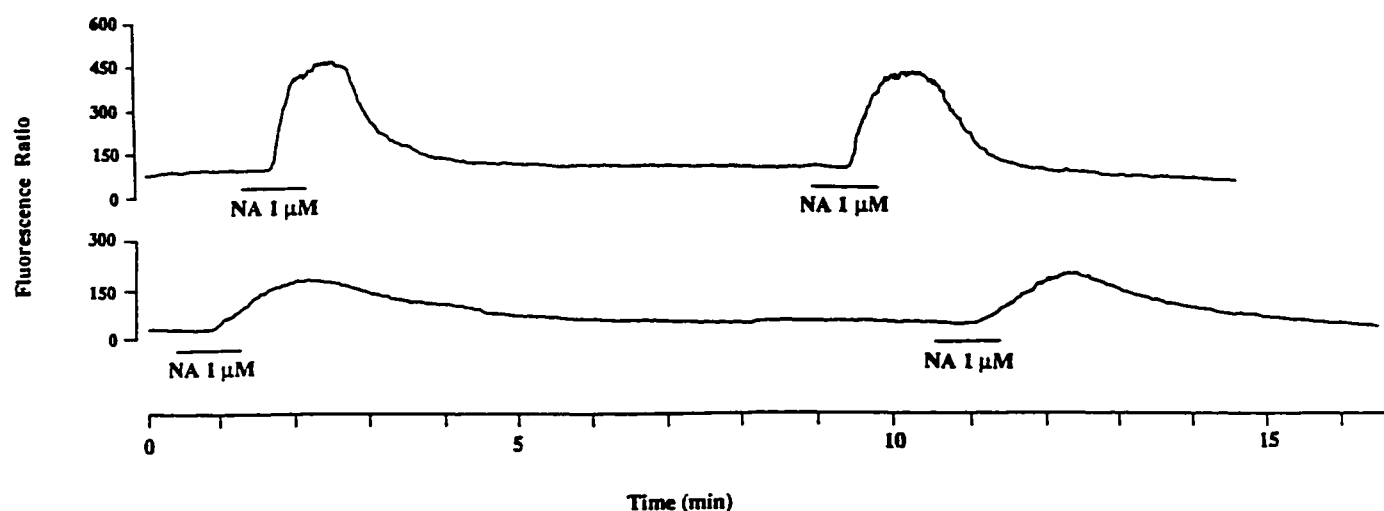


Fig. 4.18. Effect of noradrenaline on $[Ca^{2+}]_i$ in lithium treated astrocytes

Tracings of $[Ca^{2+}]_i$ over about 15 min in two individual cultures of mouse astrocytes, one untreated (upper tracing) and the other (lower tracing) treated for 14 days with 1 mM lithium chloride. Both cultures were continuously superfused with a saline containing 7.5 mM glucose. During the period indicated by the bar — 1 μ M noradrenaline was added to the superfusate.

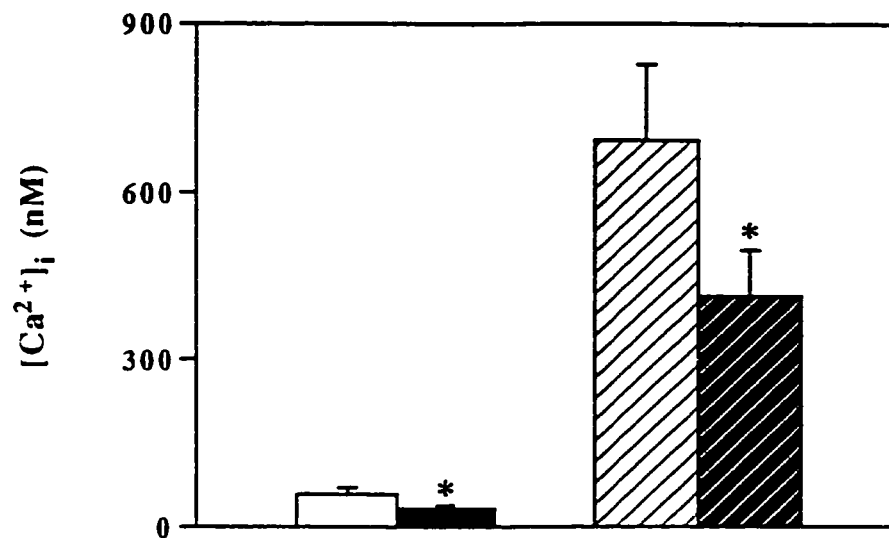


Fig. 4.19. Effect of chronic lithium treatment on noradrenaline-evoked $[Ca^{2+}]_i$ increase in astrocytes

Free cytosolic calcium concentration ($[Ca^{2+}]_i$) in primary cultures of mouse astrocytes during basal conditions (□ and ■) and during exposure to 1 μ M noradrenaline (▨ and ▩) in untreated control cultures (□ and ▨) and in sister cultures (■ and ▩) from the same batches which were studied in parallel and had been treated for 7-14 days with 1 mM lithium chloride. * indicates statistically significant difference between treated and untreated cultures. Results are means \pm S.E.M. of 13-14 individual experiments, using cultures obtained from at least 2 different batches.

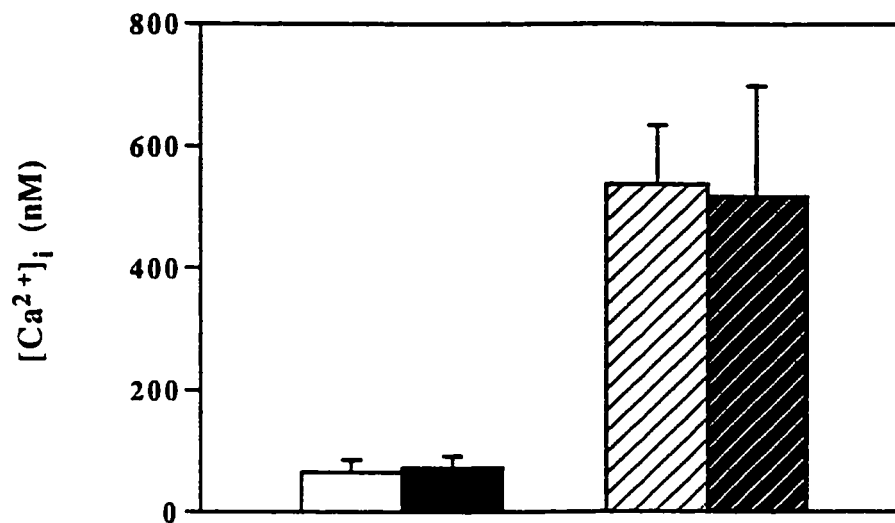


Fig. 4.20. Effect of acute lithium treatment on noradrenaline-evoked $[Ca^{2+}]_i$ increase in astrocytes

Free cytosolic calcium concentration in primary cultures of mouse astrocytes during basal conditions (□ and ■) and during exposure to 1 μ M noradrenaline (▨ and ▩) in untreated control cultures (□ and ▨) and in sister cultures (■ and ▩) from the same batches which were studied intermittently and were treated acutely (during 30 min) with 1 mM lithium chloride. In the lithium-treated and untreated cultures there was an identical increase in $[Ca^{2+}]_i$ during exposure to noradrenaline; there were no differences between the two groups of cultures either in basal $[Ca^{2+}]_i$ or during exposure to noradrenaline. Results are means \pm S.E.M. of 5-6 individual experiments, using cultures obtained from 2 different batches.

4.2. Effects of serotonergic agonists and serotonin-like drug on free cytosolic calcium and pyruvate dehydrogenation

4.2.1. Calcium response evoked by serotonin

The effect of repeated administration of the same dose of serotonin on $[Ca^{2+}]_i$ on 400/500 nm fluorescence ratio is illustrated in the recording shown in Fig. 4. 21. It can be seen that the response is identical each time the same dose is administered. This lack of desensitization allows the effect of different concentrations of serotonin or of serotonin with and without an antagonist to be studied in the same culture. The $[Ca^{2+}]_i$ response was not blocked by withdrawal of extracellular Ca^{2+} , which is in accordance with the concept that it is secondary to a release of intracellular bound Ca^{2+} (Fig. 4.22.).

According to the literature, the effects of serotonin in non-cAMP treated astrocytes require agonist concentrations at the high nanomolar or low micromolar level and are thus exerted via the 5-HT_{2A} receptor. Figure 4. 23 shows the effects of serotonin concentrations between 10 pM and 10 μ M on $[Ca^{2+}]_i$ in both dBcAMP treated astrocytes and in astrocytes that have not been treated with this compound. It can be seen that, in accordance with previous studies by other investigators, the untreated cells do not exhibit any response until the concentration of serotonin approaches about 1 μ M. However, the increase in $[Ca^{2+}]_i$ in the dBcAMP treated cells was observed at the low nanomolar and probably also at the subnanomolar level. The effect in the dBcAMP treated cells is significant from 1 nM and onward. The apparent minute stimulation at 10^{-11} M in untreated cells is not statistically significant, but the stimulation at 1 μ M is. The effect of a low concentration of serotonin (10 nM) was unaffected by ketanserin, a selective 5-HT_{2A} antagonist (Fig. 4. 24.). Together, these observations strongly suggest that the receptor involved in the effect at the low

concentrations (low nanomolar), is not the 5-HT_{2A} receptor but rather the 5-HT_{2C} receptor.

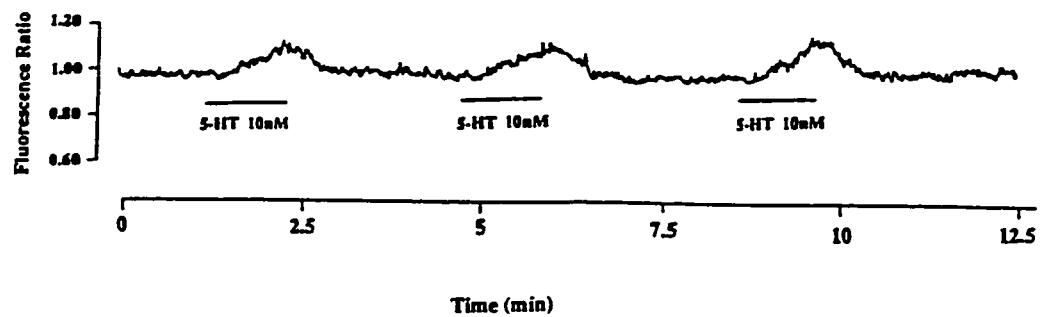


Fig. 4.21. Repeated exposure to serotonin

Changes in ratio of Indo-1/AM fluorescence at 400 and 500 nm in primary cultures of astrocytes as an indication of changes in $[Ca^{2+}]_i$ during repeated exposure to 10 nM serotonin.

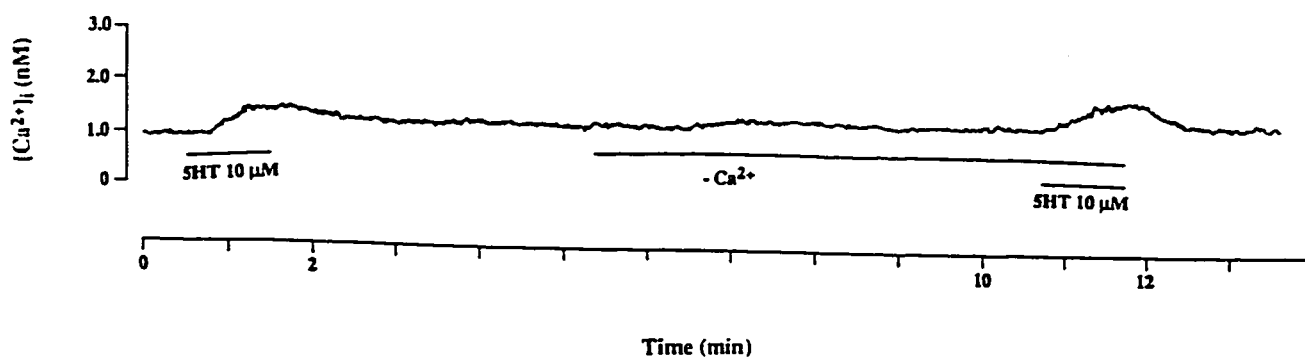


Fig. 4.22. Increase in $[Ca^{2+}]_i$ during exposure to serotonin in the presence or absence of extracellular calcium

A tracing showing the effect of extracellular Ca^{2+} on serotonin-stimulated $[Ca^{2+}]_i$ increase. Cells were initially perfused with 10 μ M serotonin (5HT) to establish a control response. During the last min of a 5 min perfusion with calcium-free medium 10 μ M of serotonin was added.

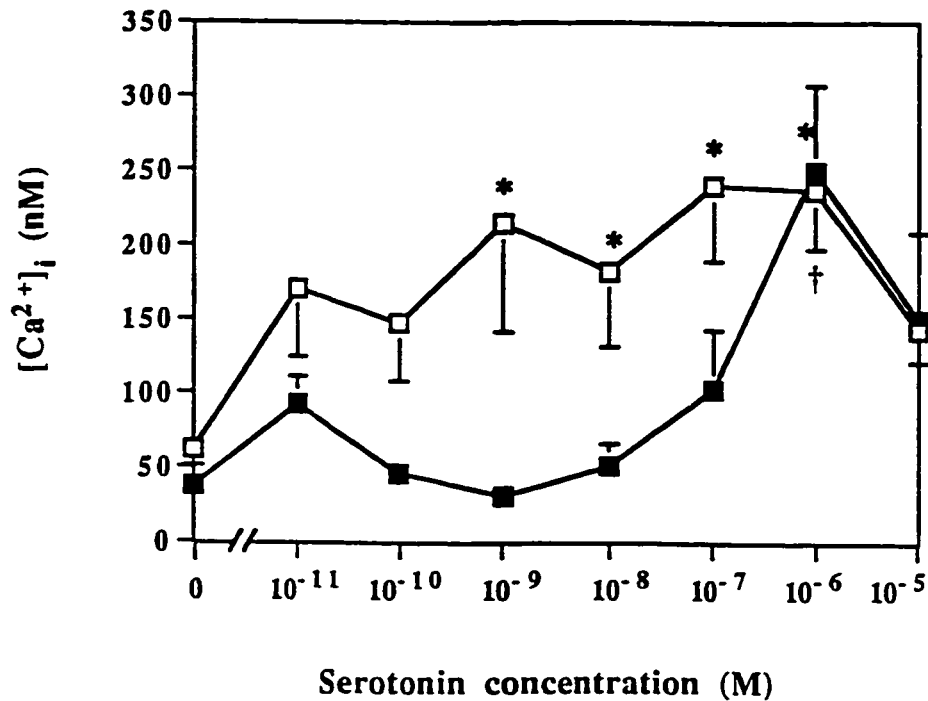


Fig. 4.23. Concentration-effect relationship between serotonin concentrations and $[Ca^{2+}]_i$ in astrocyte cultures

Effect of serotonin concentrations between 10 pM and 10 μ M on $[Ca^{2+}]_i$ in astrocyte cultures that had been treated with the differentiating compound dBcAMP (□) and in untreated cultures (■). Values significantly different from baseline (no serotonin) are indicated by * in treated cultures and by † in untreated cultures. The difference between treated and untreated cultures is significant if results for the concentrations 10 pM to 10 nM are combined. Results are averages \pm S.E.M. of 6 to 25 (treated cultures) or 1 to 3 individual (untreated cultures) experiments, and in the case of treated cells, cultures from at least two different batches.

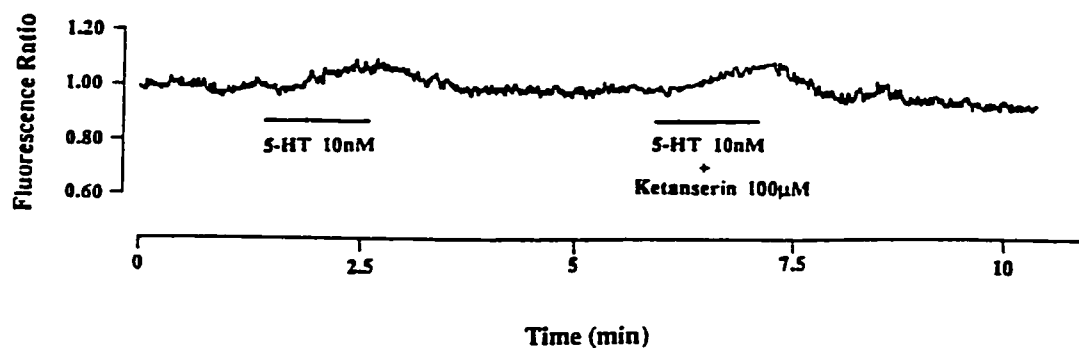


Fig.4.24. Effect of ketanserin on 10 nM serotonin-induced changes in $[Ca^{2+}]_i$ in astrocytes

Changes in ratio of Indo-1/AM fluorescence as an indication of changes in $[Ca^{2+}]_i$ during exposure to 10 nM serotonin alone and 10 nM serotonin together with 100 μ M ketanserin.

4.2.2. Calcium response evoked by fluoxetine

It is generally believed that the antidepressant drug fluoxetine exerts all its effects by inhibition of serotonin uptake into neurons resulting in increase in the extracellular concentration of serotonin. However, administration of 10 μM fluoxetine caused an increase in $[\text{Ca}^{2+}]_i$ from a resting level of about 100 nM to about 200 nM (Fig. 4.25.).

The observed increase in $[\text{Ca}^{2+}]_i$ could arise from an external source and/or a release of calcium from internal stores. Experiments during short-lasting withdrawal of extracellular Ca^{2+} were conducted to investigate the source of Ca^{2+} increase during fluoxetine stimulation. Fluoxetine was prepared in perfusion medium and cells exposed to it by change of perfusion medium. One minute later, the medium was changed back to normal control medium. When fluoxetine subsequently was administered during shortlasting calcium depletion, the normal perfusion medium was replaced with a calcium-free medium (containing zero Ca^{2+} plus 0.5 mM EGTA) during a one minute period immediately preceding the administration of fluoxetine in calcium-free medium. It can be seen from figure 4. 25. that depletion of calcium did not alter the $[\text{Ca}^{2+}]_i$ response to fluoxetine. A dose response study shows that fluoxetine concentration required to obtain $[\text{Ca}^{2+}]_i$ response is about 1 μM (Fig. 4.26.).

A fluoxetine concentration of 10 μM was chosen for the study of chronic effects of fluoxetine on $[\text{Ca}^{2+}]_i$. Other cultures from the same batches were left untreated as drug-naïve controls. After 1 week (5-7 days) of exposure to fluoxetine at this concentration and subsequent removal of the drug for at least 30 min, acute re-exposure to 10 μM fluoxetine did not significantly increase $[\text{Ca}^{2+}]_i$ (Fig. 4.27 A). This is probably not a result of a residual effect of fluoxetine since there was a non-significant

tendency towards a decrease, rather than an increase, in resting $[Ca^{2+}]_i$ in the chronically treated cultures. Drug-naïve controls from the same batch of cultures showed a normal response to acute administration of fluoxetine (Fig. 4. 27 B). In contrast, the response to 10 μ M serotonin was not down-regulated after corresponding chronic exposure to fluoxetine (Fig. 4.28 B). Drug-naïve controls from the same batch of cultures showed a normal response to acute administration of serotonin (Fig. 4. 28 A). The effect of 1 μ M serotonin on $[Ca^{2+}]_i$ was not reduced after chronic treatment with fluoxetine, whereas that of 10 nM serotonin was decreased after fluoxetine treatment (for typical recordings, see Fig. 4.29.). In experiments where 10 nM and 1 μ M were sequentially and repeatedly applied to the same culture, the fluorescence ratio change evoked by administration of 10 nM relative to that evoked by 1 μ M was significantly higher in the untreated (1.69 ± 0.13) than in the treated (0.87 ± 0.19) cultures (9 to 11 readings in 3 cultures in each group).

4.2.3. Effect of CO₂ production from [1-¹⁴C]pyruvate by serotonin and fluoxetine

Although both serotonin and fluoxetine caused an increase in $[Ca^{2+}]_i$ by releasing Ca^{2+} from intracellular Ca^{2+} store, the effects on pyruvate dehydrogenation are different. From Figure 4.30, it can be seen that serotonin had no significant effect on pyruvate dehydrogenation, whereas fluoxetine decreased CO₂ production from [1-¹⁴C] pyruvate.

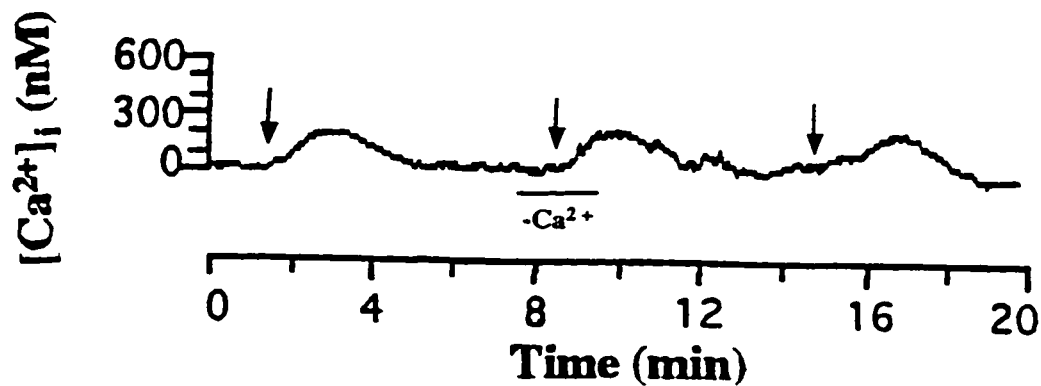


Fig. 4.25. Increase in $[Ca^{2+}]_i$ by fluoxetine in the presence or absence of extracellular calcium

Astrocytes were first exposed to 10 μ M fluoxetine, which is indicated by an arrow. One minute later the medium was changed back to normal control medium. When fluoxetine was administered during short lasting calcium depletion, the normal perfusion medium was replaced with a calcium-free perfusion medium during a 1 min period immediately preceding the administration of the drug in calcium-free medium. Then fluoxetine was added to cells again for 1 minute.

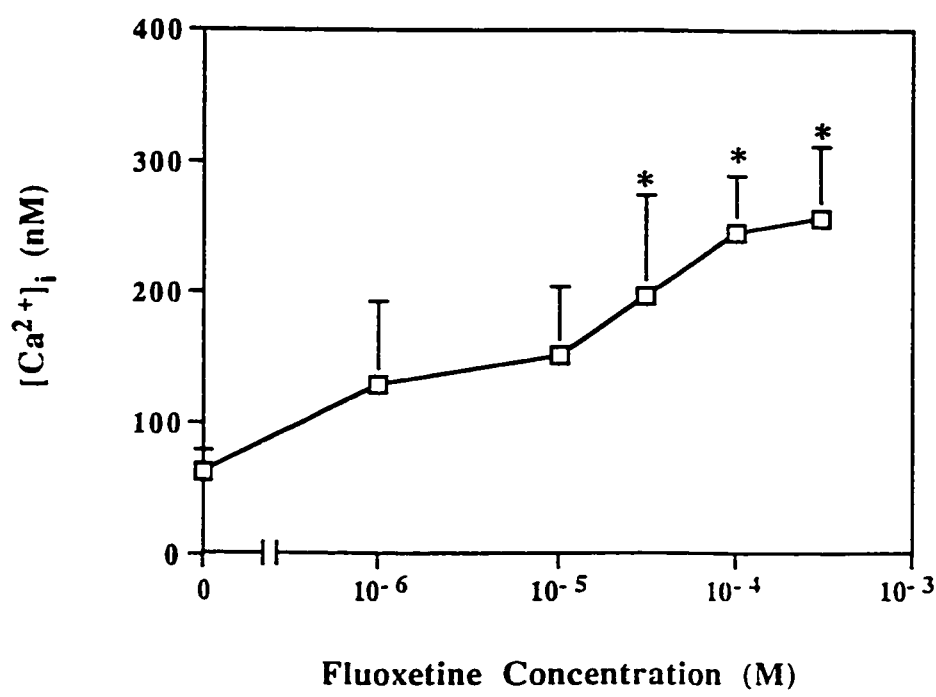


Fig. 4.26. Concentration-response relationship of fluoxetine concentrations between 1 μ M and 100 μ M on $[Ca^{2+}]_i$

A dose response curve showing the effect of fluoxetine on $[Ca^{2+}]_i$ in cultured astrocytes. * Values are significantly different from baseline (no fluoxetine). Results are average \pm S.E.M. of 3-6 individual experiments from at least two different batches.

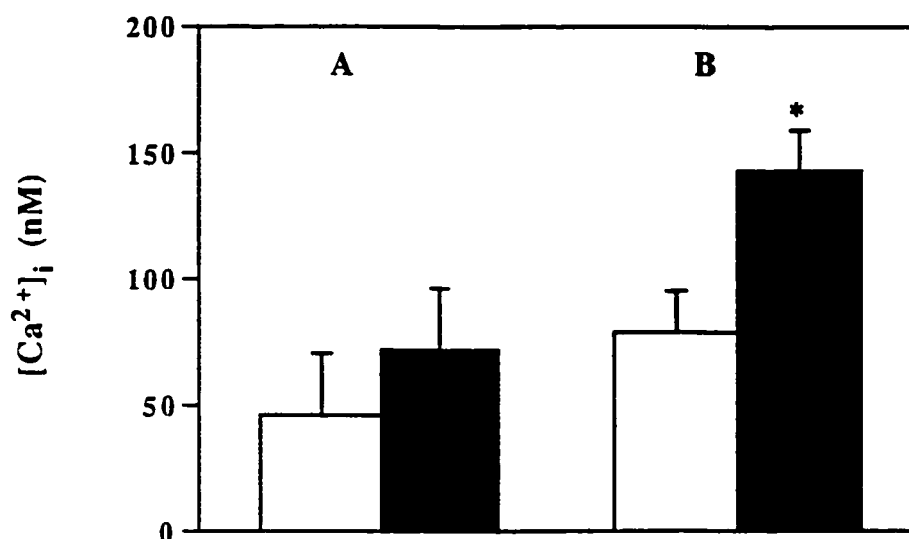


Fig. 4.27. Effect of fluoxetine on $[Ca^{2+}]_i$ in chronic fluoxetine-treated astrocytes

Astrocytes had previously been grown for 1 week in the presence of 10 μ M of fluoxetine (A) compared to the effect of acute exposure to 10 μ M fluoxetine on $[Ca^{2+}]_i$ in drug naïve cultures from the same batches (B). Resting values are indicated by (□) and values in the presence of fluoxetine by (■). Results are average \pm S.E.M. of 4-5 individual experiments using cultures from two different batches. * denotes the value is significantly different from the corresponding control value ($P < 0.05$).

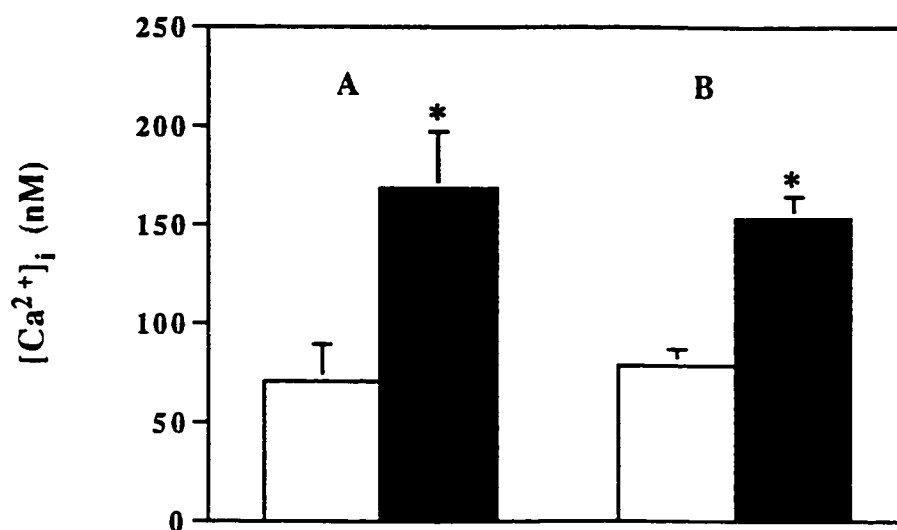


Fig. 4.28. Effect of 10 μ M serotonin on $[Ca^{2+}]_i$ in chronic fluoxetine-treated astrocytes

Astrocytes had previously been cultured for one week in the presence of 10 μ M of fluoxetine (A) compared to the effect of acute exposure to 10 μ M serotonin on $[Ca^{2+}]_i$ in drug naive cultures from the same batch (B). Resting values are indicated by (\square) and values in the presence of serotonin by (\blacksquare). Results are average \pm S.E.M. of 5 individual experiments, using cultures from two different batches. * these values are significantly different ($P < 0.05$) from the corresponding control values.

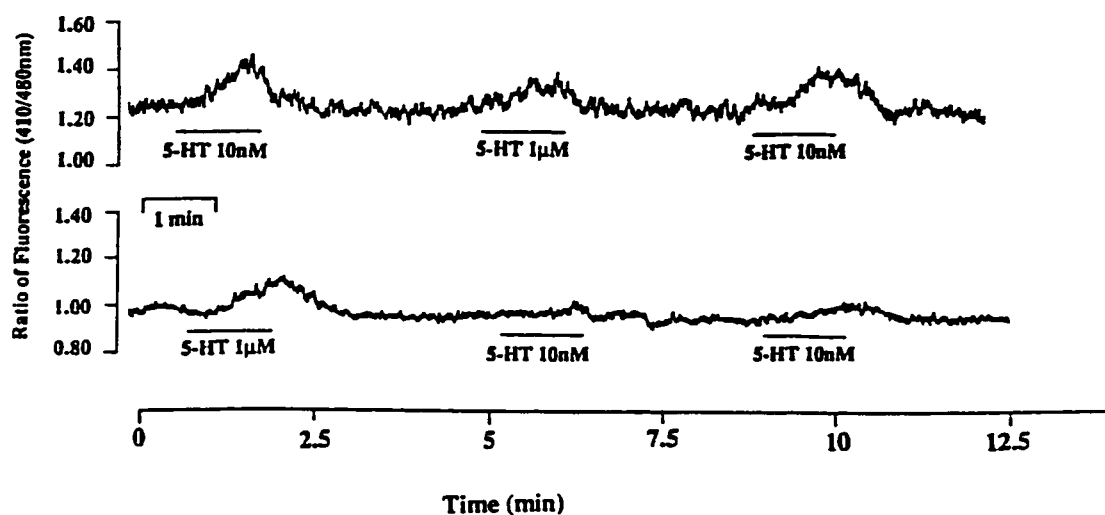


Fig. 4.29. Effect of 10 nM serotonin on $[Ca^{2+}]_i$ in chronic fluoxetine-treated astrocytes

Changes in ratio of Indo-1/AM fluorescence as an indication of changes in $[Ca^{2+}]_i$ during exposure to 10 nM and to 1 μ M serotonin concentrations in typical experiments with either a drug-naïve culture (top tracing) or a culture that had been treated for 8 days with 10 μ M fluoxetine (bottom tracing).

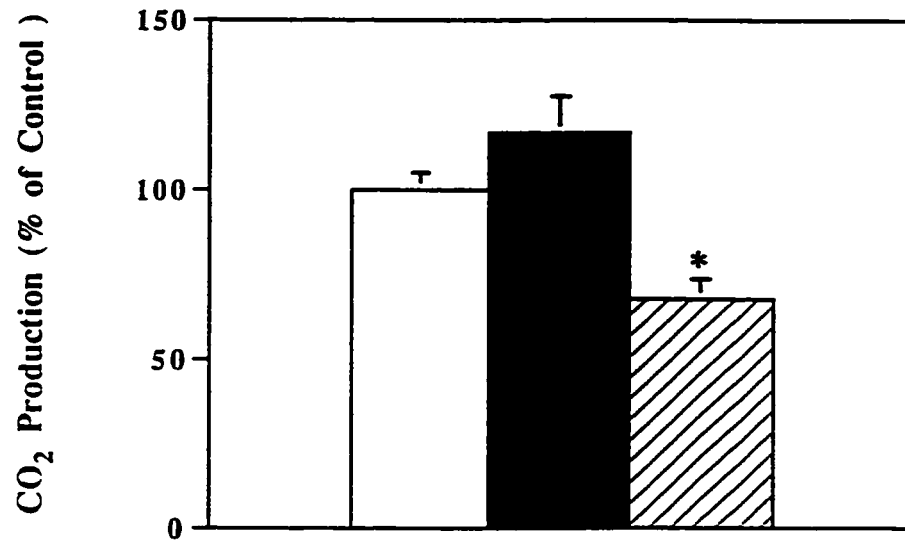


Fig.4.30. Effects of serotonin and fluoxetine on CO₂ formation from [1-¹⁴C]pyruvate

Astrocytes were incubated for 30 min with 10 μ M of serotonin (■) or fluoxetine (▨). Values are expressed as % of control (□, CO₂ formation in the absence of any serotonergic agonists). Each value represents the average \pm 6 individual experiments from 2 batches of cultured astrocytes. * this value is significantly different ($P < 0.05$) from the control.

4.3. Effects of purinergic agonists on free cytosolic calcium

Adenosine-induced increase in $[Ca^{2+}]_i$ was observed in cultured astrocytes. Maximum increases appeared with 10-100 μM , when $[Ca^{2+}]_i$ increased from a resting level of about 100 nM to about 650 nM (Fig. 4.31.). Guanosine also induced an increase in $[Ca^{2+}]_i$. A dose-response curve is presented in Fig. 4. 32. and shows that guanosine caused an increase in $[Ca^{2+}]_i$ at a concentration as low as 1 μM . The increase was maximal at about 10 μM of guanosine. 1,3-Dipropyl-7-methylxanthine (DPMX) and CGS15943, both P_1 purinergic receptor antagonists, were ineffective in blocking guanosine or adenosine stimulated $[Ca^{2+}]_i$ increase separately (Fig. 4.33 and 4.34.), indicating that adenosine and guanosine did not increase $[Ca^{2+}]_i$ by activating a P_1 -like receptor. The adenosine response underwent desensitization during repeated application of adenosine. The desensitization towards adenosine did not lead to a concomitant decrease in the response to guanosine on the same culture (Fig. 4.35.). This suggests that the two nucleosides activate separate receptors.

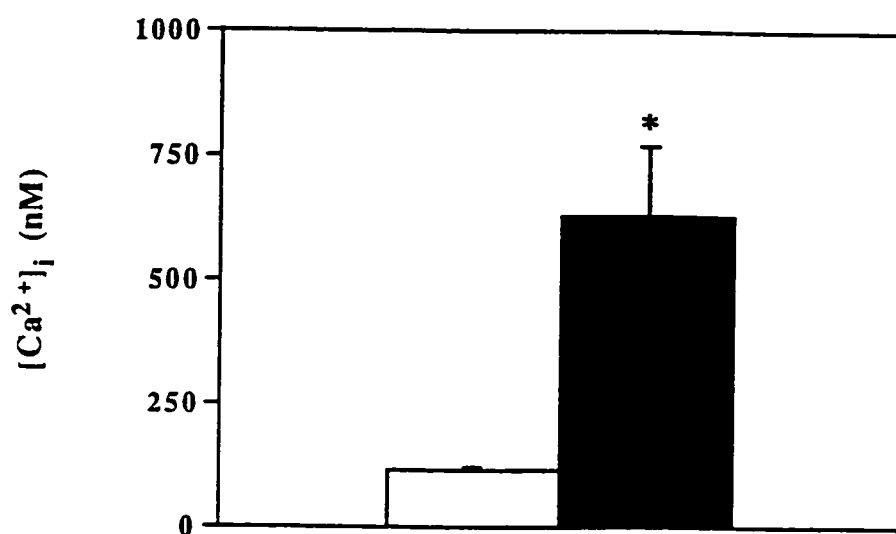


Fig. 4.31. Effect of adenosine on $[Ca^{2+}]_i$ in cultured astrocytes

10 μ M of adenosine caused an increase in $[Ca^{2+}]_i$ from 100 nM of control (□) to about 650 nM (■). Columns represent the means \pm S.E.M. of 6 individual experiments, using cultures from 2 different batches. * denotes $P < 0.05$ vs. control value.

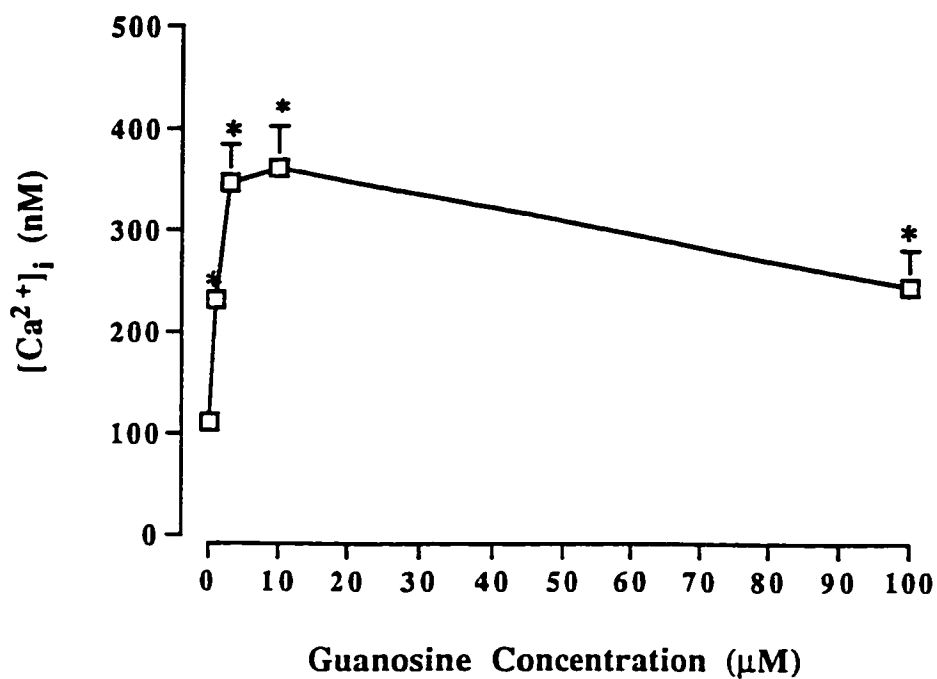


Fig. 4.32. Concentration-response relationship for guanosine and $[Ca^{2+}]_i$

The effect of guanosine on $[Ca^{2+}]_i$ in cultured astrocytes. Values are means \pm S.E.M. of 2 (1,3 μ M guanosine), 8 (10 μ M), 5 (100 μ M) and 13 (control, without guanosine) individual experiments. All values are significantly different from control ($p < 0.01$).

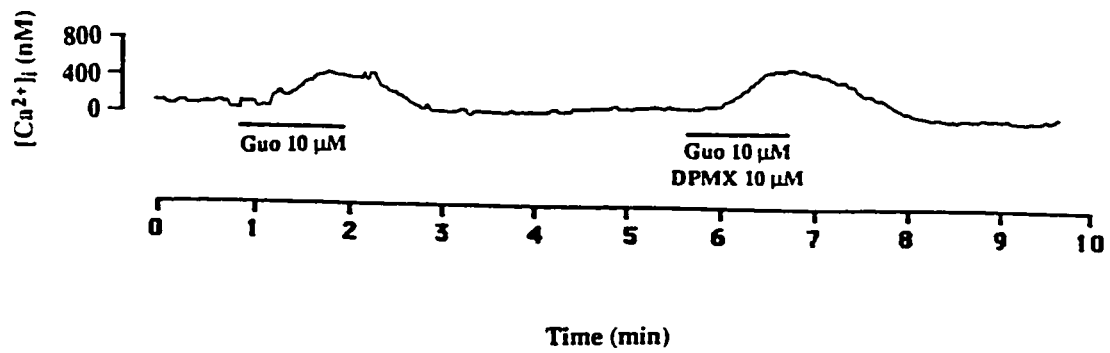


Fig.4.33. Lack effect of DPMX on guanosine-induced $[Ca^{2+}]_i$ increase

A recording showing that 10 μ M DPMX has no inhibitory effect on guanosine (Guo) evoked $[Ca^{2+}]_i$ response in cultured astrocytes.

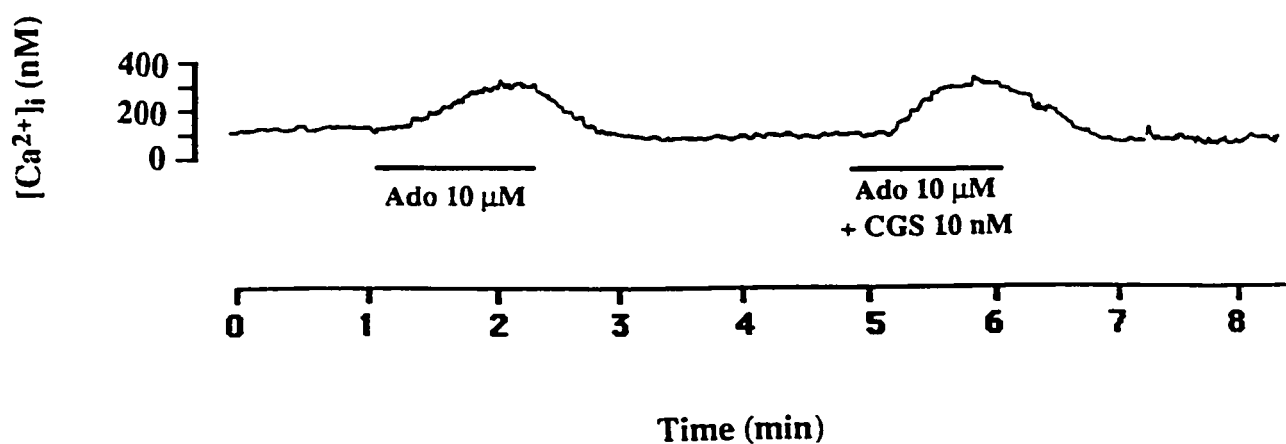


Fig.4.34. Lack effect of CGS on adenosine-stimulated $[Ca^{2+}]_i$ response

A recording showing that P_1 antagonist CGS is ineffective in preventing adenosine (Ado) induced Ca^{2+} increase in cultured astrocytes.

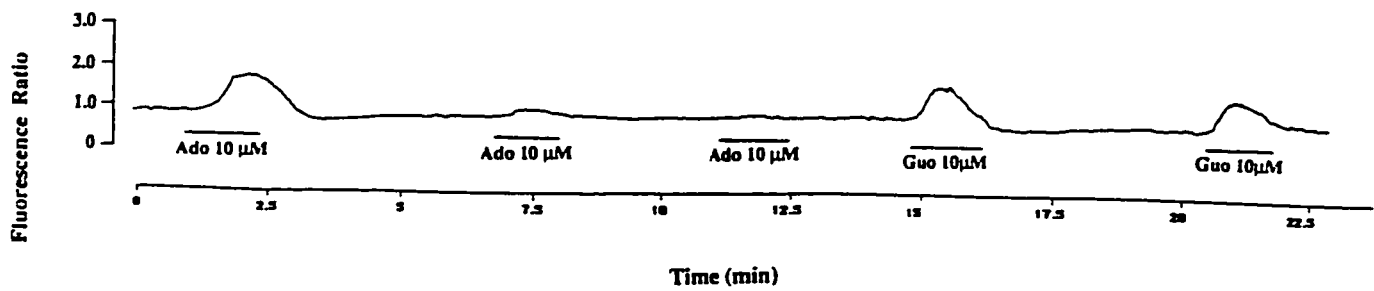


Fig.4.35. Effects of adenosine and guanosine on the same culture

The adenosine response underwent desensitization during repeated application of adenosine. The desensitization towards adenosine did not lead to a concomitant decrease in the response to guanosine on the same culture.

4.4. Effects of the peptidergic agonist vasopressin on free cytosolic calcium and pyruvate dehydrogenation

4.4.1. Calcium response evoked by vasopressin

A tracing of $[Ca^{2+}]_i$ in astrocytes during exposure to vasopressin is shown in figure 4.36. In the absence of any added drug, $[Ca^{2+}]_i$ was close to 100 nM and it increased to approximately 300 nM in the presence of 10^{-7} vasopressin regardless whether or not Ca^{2+} had been depleted from the superfusion medium shortly before the exposure to vasopressin. A concentration response relationship is shown in Figure 4.37. Since the response to vasopressin was unaltered by Ca^{2+} depletion at all vasopressin concentrations tested, results obtained in the presence and absence of Ca^{2+} were combined. Vasopressin concentrations between 10^{-9} and 10^{-6} M consistently caused an increase in $[Ca^{2+}]_i$. There was a considerable variability between individual cultures and between batches of cultures but the effect was statistically significant at 10^{-7} and 10^{-6} M.

The vasopressin-induced increase in $[Ca^{2+}]_i$ was almost abolished in the presence of the 2.5 μ M V_1 -selective antagonist d(CH)₅Tyr(me)-AVP (the Manning compound) but reappeared when this compound was again deleted (Fig. 4.38.). This observation is in agreement with the vasopressin effect being exerted on a V_1 receptor.

4.4.2. Effect of vasopressin on pyruvate dehydrogenation

Vasopressin has no significant effect on CO_2 production from $[1-^{14}C]$ pyruvate (Fig. 4.39.).

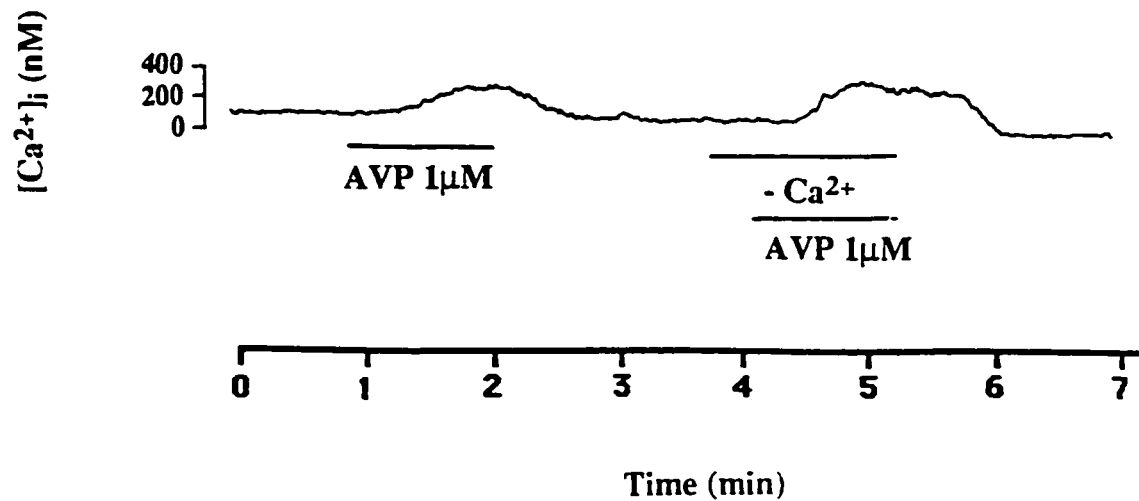


Fig. 4. 36. Effect of AVP on $[Ca^{2+}]_i$ and of removal of external calcium during AVP exposure

A representative tracing showing AVP-stimulated changes in $[Ca^{2+}]_i$ in the presence and absence of extracellular calcium. Cells were initially exposed to 1 μ M AVP, followed by 1 min superfusion with buffer containing zero Ca^{2+} plus 0.5 mM EGTA before exposure to AVP again. A similar result was seen in one other experiment.

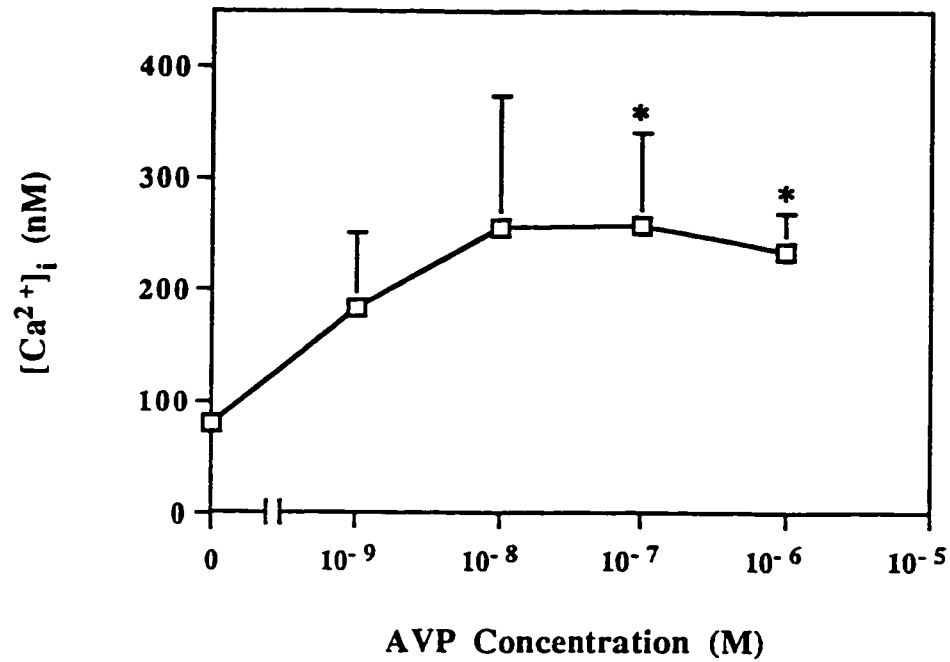


Fig. 4.37. The relation between the concentration of AVP and the increase in $[Ca^{2+}]_i$

AVP increased $[Ca^{2+}]_i$ in astrocytes over the concentration range 10^{-9} to 10^{-6} M. The effect was statistically significant at 10^{-7} and 10^{-6} M. Each data point is the mean \pm S.E.M. of 3-11 separate determinations from at least 2 batches of cultured astrocytes.

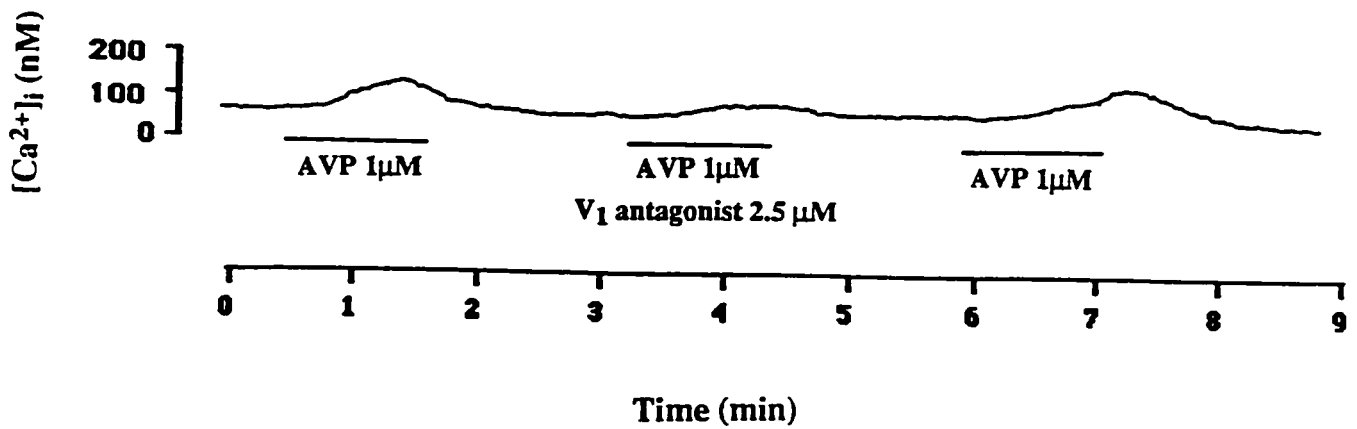


Fig. 4.38. Effect of V_1 antagonist on AVP-induced $[Ca^{2+}]_i$ response in astrocytes

A typical tracing depicts inhibition of response to 10^{-6} M AVP when 2.5 μ M V_1 antagonist was included, whereas in the absence of the V_1 antagonist, normal AVP response was elicited. One other independent experiment yielded similar results.

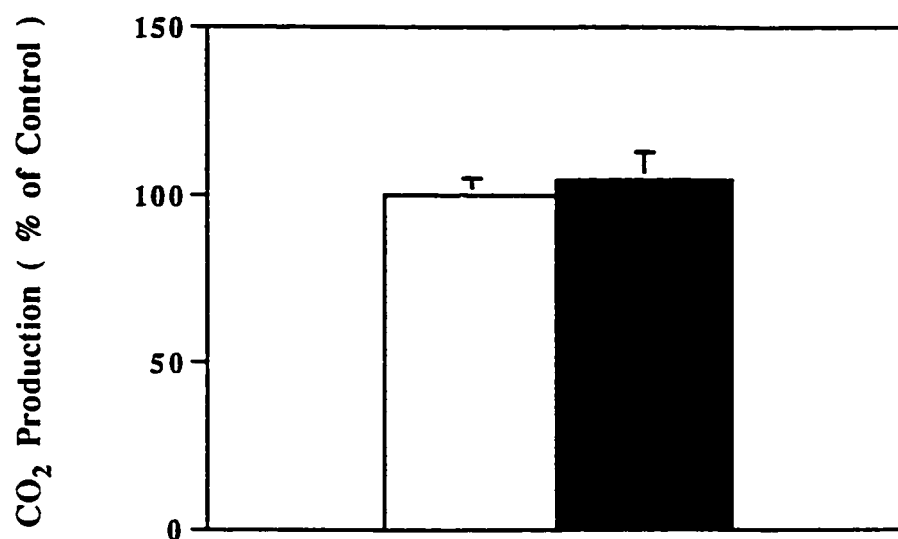


Fig. 4.39. Effect of vasopressin on CO₂ production from [1-¹⁴C]pyruvate

Cells were incubated for 30 min in the absence (□) or presence of 10⁻⁶ M AVP (■). Values are expressed as % of control. Each value represents the average ± S.E.M. of 6 individual experiments from 2 batches of cultured astrocytes.

5. DISCUSSION

5.1. Purpose of study

The purpose of the present study has been to acquire information about effects of selected representatives of three different groups of transmitters (monoamines, purines and peptide transmitters) on $[Ca^{2+}]_i$ and pyruvate dehydrogenation in astrocytes. Receptor sites for the selected transmitters are known to exist on glial cells, and studies on other tissues have indicated that pyruvate dehydrogenation is stimulated by an increase in intramitochondrial Ca^{2+} , which is triggered by a rise in $[Ca^{2+}]_i$. A major effort was made to obtain evidence for or against a correlation between transmitter effects on each of the two parameters studied and a somewhat minor effort was made to distinguish between subtypes of receptors for the transmitters. The latter aspect is complicated by the often relative, rather than absolute, selectivity of available "subtype-specific" agonists and antagonists. On account of this relative specificity it would, in many cases, have been desirable to carry out a multitude of experiments, using different combinations of agonist and antagonist concentrations. This would have been too time consuming to be possible within the framework of a Ph.D. thesis and still be able to cover the broader aspect sketched above. I have chosen to investigate the broader aspects which I believe may provide leads for further investigations. Some studies were made of drug effects. The drugs investigated either appear to be agonists themselves (fluoxetine and dexmedetomidine) or to interfere with the effect of a transmitter on $[Ca^{2+}]_i$ (lithium). This part was included in the thesis work in an attempt to make it more directly relevant for interpretation of the behavioral role of astrocytic receptor stimulation.

5.2. Effects of adrenergic agonists on $[Ca^{2+}]_i$ and pyruvate metabolism

5.2.1. Adrenergic agonists induced increase in $[Ca^{2+}]_i$ and stimulation of pyruvate dehydrogenation in primary cultures of astrocytes: Evidence for direct calcium-induced stimulation of mitochondrial pyruvate dehydrogenase activity

Noradrenergic fibers originating from the locus ceruleus in the brain stem, spread within the cerebrum, hippocampus and cerebellum and to a large extent terminate in "varicosities" rather than forming genuine synapses (Lindvall and Bjorklund, 1984). Noradrenaline released from these varicosities probably exerts a large part of its effects on astrocytes (Hertz and Hertz, 1995). A series of studies by several groups have shown that α_1 and α_2 -adrenergic agonists stimulate increases in free intracellular calcium concentration in cultured astrocytes (Salm and McCarthy, 1990; Delumeau, et al., 1991; Nilsson, et al., 1991b). Using the Indo-1 method, a highly sensitive procedure to detect changes in intracellular free calcium levels, the present study further confirms that stimulation of α -adrenoceptors by noradrenaline causes a rise in cytosolic Ca^{2+} ($[Ca^{2+}]_i$). This effect can be evoked both by α_1 -adrenergic and α_2 -adrenergic receptor activation, since the selective α_1 -adrenergic agonist, phenylephrine, and the selective α_2 -adrenergic agonist, clonidine, both induce $[Ca^{2+}]_i$ increase. Noradrenaline and phenylephrine induced $[Ca^{2+}]_i$ responses were abolished completely by phentolamine, an α -adrenergic (both α_1 and α_2) antagonist. Moreover, the clonidine induced response was inhibited by yohimbine. Thus, both agonist and antagonist studies indicate that α_1 -adrenergic as well as α_2 -adrenergic stimulation causes an increase in $[Ca^{2+}]_i$. As could be expected, β -adrenergic stimulation exerts no such effect.

α_2 -Adrenergic receptors are generally regarded as being presynaptically located and inhibiting noradrenaline release upon stimulation. The first indication that α_2 agonists can cause an increase of cytosolic calcium concentration was found in smooth muscle (Jim and Matthews, 1985). This response was interpreted as being due to activation of postsynaptic receptors. The present results indicate that clonidine has a similar effect on $[Ca^{2+}]_i$ in astrocytes through postsynaptic α_2 adrenoceptors.

McCarthy and Salm (1991) have reported that over 80% of astrocytes in primary cultures responded to noradrenaline stimulation with an increase in $[Ca^{2+}]_i$. Although more cells appeared to react to α_1 -adrenergic than to α_2 -adrenergic receptor activation, some cells responded equally well to activation of either subtype receptor, and there was a subpopulation of cells which responded only to stimulation of α_2 -receptors. This appears to be consistent with our findings that noradrenaline is more effective than clonidine in causing an increase in $[Ca^{2+}]_i$.

The metabolism of pyruvate is an especially pivotal step in glucose degradation. Utilization of glucose-derived acetyl-CoA in the TCA cycle is by far the most important pathway for oxidative energy production in brain (Sokoloff, 1992). Therefore, it is important that formation of acetyl-CoA from pyruvate also is enhanced by noradrenaline.

The present study showed that clonidine exerts a stimulation on pyruvate dehydrogenation and there was a tendency towards a small stimulation by phenylephrine, whereas isoproterenol had no effect. This receptor subtype specificity was confirmed by investigating the effect of noradrenaline in the absence of any antagonist and in the presence of prazosin, yohimbine and propranolol. The stimulation

by noradrenaline was inhibited in the presence of yohimbine, and prazosin exerted a small non-significant effect, whereas no effect was found with propranolol.

It is known that events connected with an increase in $[Ca^{2+}]_i$ are largely energy-requiring; therefore the energy-producing metabolism of the cell has to be stimulated (McCormack and Denton, 1993). Although many extracellular agents, such as hormones, are known to influence mitochondrial processes, Ca^{2+} remains the only second-messenger molecule which is known to be able to cross the inner membrane and access the matrix and hence affect target proteins within this compartment (McCormack and Denton, 1986). In heart muscle and certain other tissues, noradrenaline increases pyruvate dehydrogenation and this effect is evoked by an increase in intramitochondrial Ca^{2+} which, in turn, is secondary to an increase in cytosolic Ca^{2+} (McCormack and Denton, 1993).

The enhancement of pyruvate dehydrogenation in astrocytes might be evoked by mechanism similar to that in heart muscle. Evidence for a correlation between metabolic stimulation and intramitochondrial concentration of calcium is that omission of extracellular calcium, together with an elevation in the magnesium content, a procedure known to inhibit mitochondrial calcium uptake, abolished the stimulation of $^{14}CO_2$ formation from $[1-^{14}C]$ pyruvate by noradrenaline. Moreover, after one hour incubation in PBS containing nominally "zero" calcium, the NA induced stimulation of flux from pyruvate to acetyl-CoA was abolished. However, the stimulation was not inhibited by ruthenium red. The reason for this may be that ruthenium red was simply not taken up into the cells. This question requires further investigation.

Although excess potassium increases many aspects of energy metabolism, such as lactate production, oxygen consumption and glycogenolysis, CO_2 fixation (Hertz and

Peng, 1992b; Kaufman and Driscoll, 1992), as well as free intracellular calcium concentration (Code et al., 1992), it did not increase the flux through the pyruvate dehydrogenase catalyzed step (CO_2 formation from $[1-^{14}\text{C}]$ pyruvate) in the present study. An elevated potassium concentration also did not stimulate the α -ketoglutarate dehydrogenase catalyzed CO_2 production from glutamate in intact astrocytes (Hertz and Peng, 1992b). Moreover, some evidence has indicated that the depolarization-induced stimulation of energy metabolism in synaptosomes is not caused by calcium induced intramitochondrial effects, but rather is the result of an altered ADP/ATP ratio (Hansford and Castro, 1985; Hansford, 1988; Kauppinen et al., 1989), in accordance with the classical Chance-Williams model (Chance and Williams, 1956). Therefore, it should be emphasized that not every increase in $[\text{Ca}^{2+}]_i$ is correlated with a metabolic increase due to direct calcium effects on the dehydrogenases. This conclusion is strengthened by the finding that phenylephrine, serotonin and vasopressin all increased $[\text{Ca}^{2+}]_i$ but did not stimulate pyruvate dehydrogenation. This distinction between transmitter effects on $[\text{Ca}^{2+}]_i$ and on pyruvate dehydrogenation is not unusual since $[\text{Ca}^{2+}]_i$ may increase in many different parts of the cells and be an intracellular messenger for more than one process.

5.2.2. Dexmedetomidine, an α -adrenergic agonist, increased free cytosolic calcium concentration and pyruvate metabolism in astrocytes

Dexmedetomidine is a highly specific, potent α_2 -adrenergic agonist, used in anesthesia because of its capability to potentiate the effect of volatile and opioid-like anesthetics. At higher concentrations, it has sedative-hypnotic effects on its own. The present study has shown that dexmedetomidine resembles clonidine and noradrenaline in causing an increase in $[\text{Ca}^{2+}]_i$ and pyruvate dehydrogenation in astrocytes.

The maximum increase in $[Ca^{2+}]_i$ at 70 nM dexmedetomidine is similar to that previously demonstrated close to this concentration (Zhao et al., 1992), and a second peak of about the same magnitude was observed at 10 to 25 μM . This effect was inhibited both by yohimbine, the archetypical α_2 -adrenergic antagonist, and by idazoxan, an antagonist of the imidazoline preferring receptor, suggesting that it may be related to the clinically used effect of this drug, which is also abolished by either yohimbine or idazoxan (Meert and De-Kock, 1994).

There is an identical concentration dependence of the dexmedetomidine effect on pyruvate dehydrogenation. The dexmedetomidine effect on pyruvate oxidation also resembles the effect on $[Ca^{2+}]_i$ by being inhibited by both yohimbine and idazoxan. This effect may therefore be directly correlated with the increase in $[Ca^{2+}]_i$ and the potentiation of anesthetics by dexmedetomidine.

5.2.3. Inhibition of noradrenaline stimulated increase in free cytosolic calcium concentration in astrocytes by chronic treatment with lithium

It has been suggested that lithium ion exerts its antidepressant effect by inhibiting inositol monophosphatase (Hallcher and Sherman, 1980), leading to build-up of inositol-1-phosphate, depletion of inositol, interference with the phosphatidylinositol (PI) cycle (Berridge et al., 1989), and reduction of the production of the two secondary messengers DAG and IP_3 . In this study, a reduction of noradrenaline-evoked $[Ca^{2+}]_i$ response by chronic treatment with lithium, is consistent with the expected effect of an impairment of turnover in the PI cycle. It does not exclude an involvement of other potential actions of lithium, e.g., a direct action on G proteins, but chronic exposure to lithium does not appear to affect G proteins linked to phosphoinositol turnover (Manji, et al., 1995).

Astrocytes appear to be a target for antidepressant drugs, since chronic, but not acute, exposure to a tricyclic antidepressant or a monoamine oxidase inhibitor decreases isoproterenol induced stimulation of the formation of cyclic AMP in astrocytes (Hertz and Richardson, 1983), i.e., a reduction of a noradrenergic response. Thus, several different groups of antidepressant drugs appear to downregulate noradrenergic responses in astrocytes with a time course paralleling that of their therapeutic efficacy, suggesting that astrocytic dysfunction may play an important role in the pathophysiology of manic-depressive disorder.

5.3. Serotonin and a serotonin-like drug, fluoxetine, increase $[Ca^{2+}]_i$, interacting with a 5-HT_{2C} receptor in astrocytes

Two serotonin receptor subtypes react to stimulation of the phosphoinositol second messenger system with a resulting increase in free cytosolic concentration of calcium, i.e. the 5-HT_{1C} and the 5-HT_{2A} receptors. The former one has now been renamed the 5-HT_{2C} receptor because it functionally, molecularly, and evolutionarily shares more characteristics with the other 5-HT₂ receptors than with the 5-HT₁ family (Humphrey et al, 1993; Martin and Humphrey, 1994; Peroutka and Howell, 1994). However, serotonin actions at the 5-HT_{2A} and 5-HT_{2C} receptors can be distinguished on the basis of a high affinity of the 5-HT_{2C} subtype for serotonin versus a low affinity of the 5-HT_{2A} subtype (Peroutka and Snyder, 1979).

The 5-HT receptor known from studies by two different groups to be expressed by astrocytes is the 5-HT_{2A} receptor (Deecher et al., 1993; Nilsson et al., 1991b), which is stimulated by micromolar concentration of serotonin. The present study confirms the finding that this subtype is the only functional calcium related serotonin receptor in

astrocytes that have been cultured in the absence of dBcAMP. The magnitude of the response is identical to that observed by Deecher et al. (1993). The present study provides additional, new information that in astrocytes that have been cultured in the presence of dBcAMP, 5-HT_{2C} receptors are present rather than the 5-HT_{2A} or possibly together with 5-HT_{2A} receptor. The evidence for the existence of 5-HT_{2C} receptor in astrocytes is that the increase in $[Ca^{2+}]_i$ in the dBcAMP treated cells was observed at low nanomolar and also at the subnanomolar level. This is in agreement with the high affinity characteristic of serotonin 5-HT_{2C} subtype. This conclusion was corroborated by the observation that the effect of low concentration of serotonin (10 nM) was unaffected by ketanserin, a relatively selective 5-HT_{2A} antagonist (Pierce et al., 1992). Evidence in favor of the involvement of the 5-HT_{2C} receptors is also obtained from the study of effects of fluoxetine on glycogenolysis and free cytosolic calcium concentration in astrocytes.

Fluoxetine is generally supposed to be a specific uptake inhibitor for serotonin with no direct action on serotonin receptors, but it has been found to displace high-affinity serotonin binding from intact cultured astrocytes in culture (Hertz et al., 1979), from an astrocytic serotonin binding protein (Hertz and Tamir, 1981), and from choroid plexus cells (Leonhardt, et al., 1992). Fluoxetine increases glycogenolysis in astrocytes, an effect which is inhibited by mesulergine (an antagonist of both 5-HT_{2C} and 5-HT_{2A} receptors, which is often assumed to be 5-HT_{2C} selective) but not by ketanserine (Zhang et al., 1993), indicating that the stimulation of glycogenolysis by fluoxetine is likely to be the result of 5-HT_{2C} receptor activation. The present observation that chronic exposure to fluoxetine reduces the response to nanomolar concentrations of serotonin (acting on 5-HT_{2C} receptor) but, to a lesser extent or not at all, to micromolar concentrations of serotonin (acting on the 5-HT_{2A} receptor) supports the concept that fluoxetine interacts with the 5-HT_{2C} receptor. So do the observations that fluoxetine has

a high affinity for a cloned 5-HT_{2C} receptor (Wood et al., 1993) and a relatively high affinity for the 5-HT_{2C} binding site in brain tissue (Wong et al., 1991; Jenck et al., 1993). Also, a behavioral test has suggested that the 5-HT_{2C} receptor is the most important serotonin receptor subtype activated by administration of fluoxetine (Berendson and Broekkamp, 1994), although this response obviously could be secondary to the evoked increase in extracellular serotonin concentration.

The present study suggests a direct receptor action of fluoxetine during treatment with this drug by demonstrating that a relevant concentration of this drug in the absence of serotonin causes an increase of free cytosolic calcium concentration in astrocytes. A direct action of fluoxetine on the 5-HT_{2C} receptor would be of major functional importance because such receptors are prominent in brain (Hoffman and Mezey, 1989; Mengod et al., 1990; Molineaux et al., 1987). In the treatment of depression, antidepressant medication, including fluoxetine, must be administered for at least a couple of weeks before the therapeutic effect occurs. This study suggests that exposure of astrocytes to fluoxetine for 1 week causes a pronounced downregulation of 5-HT_{2C} receptors. The most common effect of chronic exposure of rodents to fluoxetine is downregulation of the high-affinity 5-HT receptors, which include the 5-HT_{2C} receptor (Beasley et al., 1992). The very high potency of serotonin at 5-HT_{2C} receptor sites suggests that astrocytes may be affected by serotonin released from "varicosities" even at a considerable distance from the cells. Thus, the consequences of astrocytic 5-HT_{2C} stimulation may be much more widespread than those of 5-HT_{2A} activation of these cells.

Serotonin does not affect pyruvate dehydrogenation, while fluoxetine decreases this reaction. This is further evidence that not any increase in free intracellular calcium concentration is correlated with a metabolic increase due to direct calcium effects on the

dehydrogenases. However, an increase in glycogenolysis by serotonin or fluoxetine might be secondary to the increase in $[Ca^{2+}]_i$ (Hertz and Code, 1993; Zhang et al., 1993). The mechanism by which fluoxetine decreases pyruvate dehydrogenation is unknown, but it should be mentioned that, in contrast to noradrenaline, serotonin decreases glucose utilization and oxygen consumption in the brain in vivo (MacKenzie et al., 1977; Grome and Harper, 1985).

5.4. The effect of adenosine and guanosine on free cytosolic calcium concentration in astrocytes

Both extracellular adenosine and guanosine increased intracellular calcium concentration. This increase was not inhibited by antagonists of adenosine P_1 receptors, indicating that the effect is not due to the activation of P_1 receptors. Since there are no specific antagonists of P_2X and P_2Y purinoceptors, it could not be determined which receptor subtype is involved in increasing intracellular calcium. Some reports have indicated that stimulation of the P_2Y receptor increases inositol phosphate formation and calcium mobilization in astrocytes (Kastritsis et al., 1992).

Adenosine and guanosine are known to stimulate proliferation in astrocytes and the effect is correlated to cAMP increases (Rathbone et al., 1991). The increase in intracellular calcium concentration may also be involved in cell proliferation by activating of protein kinase C.

Guanosine has not previously been known to bind to any specific purinergic receptors (Rathbone et al., 1992). The effect demonstrated in the present study implies that guanosine might exert an effect at the cell surface, perhaps through some as yet unidentified guanosine receptor. This receptor must be different from the adenosine

receptor, since the desensitization towards adenosine did not lead to a decrease in the response to guanosine on the same culture.

5.5. Vasopressin induced increase in $[Ca^{2+}]_i$ concentration in astrocytes

The result that vasopressin causes an increase in $[Ca^{2+}]_i$ in astrocytes, indicates that the peptide acts on a V_1 receptor in these cells. Previous observations of vasopressin effects on cell volume (Chen et al., 1992) suggested that this might be the case. This was unexpected since vasopressin effects on fluid regulation generally are exerted on a V_2 receptor, operating via the adenylate cyclase system (Cornett and Dorsa, 1985). In the present study it has, however, been unequivocally confirmed that vasopressin increases $[Ca^{2+}]_i$ in astrocytes and the characterization of the receptor as a V_1 receptor was confirmed by the inhibitory effect of a V_1 -antagonist. This is consistent with the finding that vasopressin interacts with the IP_3 system in astrocytes (Cholewinski et al., 1988) and that vasopressin acts on a V_1 receptor in brain tissue (Phillips et al., 1990). Furthermore, vasopressin has previously been found to cause an increase in $[Ca^{2+}]_i$ in pituicytes, a specialized type of astrocyte and this increase could be blocked by V_1 antagonists (Hatton et al., 1992).

The concentration response correlation indicated responses between 1 nM and 1 μ M and other experiments (Zhao and Hertz, unpublished experiments) have shown a distinct effect also at 100 pM. The potency of vasopressin seen in the present study is thus higher than in the study by Hatton et al. (1992) where a minimum concentration of 10 nM was required or in the study of the effect of vasopressin on cell swelling by Latskovits et al. (1993) where no response was elicited by 1 nM. Still, the potency observed in the present study is not as high as in the previous study on the vasopressin effect on astrocyte swelling (Chen et al., 1992) where an effect was observed at 1 pM,

i.e., a concentration similar to that occurring physiologically (Luerssen and Robertson, 1980). One reason for this discrepancy may be that no attempt was made to study systematically the effects of vasopressin on $[Ca^{2+}]_i$ at lower concentrations. Another might be that the cells for the present studies have to be cultured on coverslips where they cannot be maintained as long as in tissue culture dishes and the response to vasopressin might increase with age in culture. Finally, peptide transmitters are notorious for binding to glass surfaces and might possibly also bind to the plastic tubing.

Vasopressin has no effect on pyruvate dehydrogenation. The possible physiological role of vasopressin activated IP_3 - $[Ca^{2+}]_i$ system on astrocytes remains to be elucidated, but it may be related to vasopressin effects on cell volume.

5.6. Concluding remarks

5.6.1. Contributions of present work

This study has focused on transmitter effects on calcium signalling and energy metabolism related to calcium signalling in astrocytes. All findings are summarized in Table 5.1. The findings suggest that adrenergic agonists increase the rate of oxidative metabolism in astrocytes by an action on receptors that are linked to the phosphoinositol second messenger system and therefore also cause an increase in $[Ca^{2+}]_i$. By analogy with muscle tissue, it is tempting to assume that the stimulation of energy metabolism is brought about by an increase in intramitochondrial calcium concentration, secondary to the increase in $[Ca^{2+}]_i$. However, ruthenium red, which in muscle prevents the increase in intramitochondrial calcium following a transmitter induced increase in $[Ca^{2+}]_i$ and therefore abolishes the stimulation of pyruvate dehydrogenation, had no inhibitory effect in astrocytes. The possibility of other mechanisms of action for noradrenaline

should also be considered, but it was beyond the scope of the present work to investigate any of these. Serotonergic agonists and the peptidergic transmitter vasopressin evoke a rise in $[Ca^{2+}]_i$ that is not related to pyruvate metabolism. This does not indicate that an increase in $[Ca^{2+}]_i$ could not be the only signal for stimulation of pyruvate dehydrogenation, but it does mean that increases in $[Ca^{2+}]_i$ also serve as second messenger for other astrocytic functions, for example, the serotonergic stimulation of glyconeogenesis. The findings that selected noradrenergic, serotonergic, purinergic and peptidergic agonists cause an increase in $[Ca^{2+}]_i$ in primary cultures of astrocytes through specific receptors presents further evidence that astrocytes are targets for many transmitters released from neurons. They also suggest that calcium, as a second messenger, may play a very important role in regulating astrocytic functions. Drugs interacting with these transmitter functions may exert at least part of their action on CNS function by effects on astrocytes.

In summary, returning to the hypotheses outlined in 2.1, hypotheses # 1-3 have been confirmed, i.e., noradrenaline and dexmedetomidine increased both $[Ca^{2+}]_i$ and pyruvate dehydrogenation, and lithium reduced the noradrenaline-induced increase in $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ by serotonin and fluoxetine hypothesized in # 4-5 have also been confirmed, but serotonin did not affect pyruvate dehydrogenation, and fluoxetine caused a decrease. Hypothesis # 6 has been confirmed, but it was only the response to nanomolar concentrations of serotonin which was altered, suggesting a selective downregulation of the 5-HT_{2C} receptor. The increase in $[Ca^{2+}]_i$ hypothesized in # 7-8 was confirmed, but like serotonin, vasopressin did not affect pyruvate dehydrogenation. Thus, it is only in the case of adrenergic agonists that evidence was found in favour of a direct link between $[Ca^{2+}]_i$ and pyruvate dehydrogenation. Whether such a direct correlation depends upon activation of specific second messengers is unknown. Even among adrenergic agonists the correlation was not

complete, since α_1 -adrenergic agonists increased $[Ca^{2+}]_i$ substantially, but had little if any effect on pyruvate dehydrogenation. On the other hand, a stimulation of pyruvate dehydrogenation was never seen without an increase in $[Ca^{2+}]_i$.

5.6.2. Suggestions for further studies

In "Purpose of study" it was indicated that firm establishment of transmitter subtypes will require additional work utilizing different agonist and antagonist concentrations. A different approach would be to test for the messengers of the different subtypes using molecular biological techniques. Such work also seems mandatory for a direct confirmation of the different subtypes of 5-HT receptors present on astrocytes, perhaps reflecting their degree of differentiation.

Evidence was found in the study that some of the most frequently and successfully used drugs in the treatment of mental disorders, especially affective illnesses (lithium, fluoxetine) may exert at least part of their action on astrocytes. The same may be true for the anesthetic adjuvant dexmedetomidine. Little is known about the mechanism(s) by which these drugs exert their beneficial therapeutic action and it would appear useful to pursue this avenue and obtain more information how these drugs affect, respectively, astrocytes and neurons. Especially the results for lithium indicate relevant studies which it has not been possible to include in this thesis, e.g. whether lithium also affects noradrenaline stimulation of pyruvate dehydrogenation, how it influences the phosphoinositol system in astrocytes and whether it may exert similar actions on neurons. The latter two points should also be investigated in the case of dexmedetomidine. The fluoxetine studies were the most complete of the drug studies but also these studies raise additional questions, e.g., whether other serotonin uptake inhibitors which have a different ratio between their affinity for neuronal serotonin

transporters and 5-HT_{2C} receptor (e.g. paroxetine) act differently from fluoxetine. Such studies may open the way for behavioral studies aiming at determining the relative importance of serotonin uptake inhibition and of direct stimulation of astrocytic 5-HT_{2C} receptors when these compounds are used therapeutically.

This work has dealt with influences of $[Ca^{2+}]_i$ on metabolism. A recent, fascinating study by Lechleiter and coworkers (Jouaville et al., 1995) has shown that at least in oocytes there is also a correlation in the opposite direction: Metabolic alterations affect calcium wave activity. It would be very important to know whether a similar correlation exists in astrocytes, enabling changes in metabolic intensity to alter calcium signalling, with all the potential consequences for CNS activity this may entail.

Table 5.1. Summary

Receptor	Agonist	[Ca ²⁺] _i	Pyruvate Dehydrogenation
Adrenergic			
α_1	NA, Phenylephrine	↑↑	?
α_2	NA, clonidine, Dexmedetomidine	↑↑	↑↑
β	Isoproterenol	—	—
Serotonergic			
5HT _{2A} (5HT ₂)	Serotonin	↑↑	—
5HT _{2C} (5HT _{1C})	Serotonin	↑↑	?
	Fluoxetine	↑↑	↓
Peptidergic			
AVP ₁	Vasopressin	↑↑	—
Purinergic			
P ₂ (?)	Adenosine	↑↑	?
?	Guanosine	↑↑	?

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