Functional Studies of Calbindin-D_{28K} and its Role in Intracellular Calcium Homeostasis

by

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Abstract

Calbindin-D28k (CaBP) is a 28 kD calcium-binding protein found in specific neuronal populations in the mammalian brain. The hypothesized Ca^{2+}-buffering action of CaBP is the basis of suggestions that this protein may serve to protect neurons against cell death mediated by large or prolonged increases in intracellular free Ca^{2+} concentration. However, to date, there is little direct evidence to support this hypothesis. To address this question directly, we have examined Ca^{2+}-buffering by CaBP in stably transfected HEK 293 and HeLa cell lines. A variety of methods were employed to induce calcium transients, including transfection of NMDA receptors followed by activation with glutamate. In all experiments there was evidence of CaBP-mediated Ca^{2+}-buffering. Moreover, when NMDAR transfected cells were exposed to excitotoxic concentrations of glutamate, cells expressing CaBP exhibited enhanced survival over controls. CaBP was unable to prevent acute necrotic cell death but significantly protected cells from delayed, presumably apoptotic cell death.

To examine the potential influence of CaBP upon intracellular Ca^{2+}-oscillations, stably-transfected HeLa cells were treated with histamine, while measuring intracellular Ca^{2+}. The observation that CaBP flattened the profile of component Ca^{2+} peaks, coupled with data from HEK cell lines, provides unequivocal evidence that CaBP can act to buffer increases in intracellular Ca^{2+}.

Utilizing a novel method for resolving intracellular Ca^{2+} waves, it was found that transfection with CaBP, or loading with artificial Ca^{2+} buffers, attenuated the velocity of Ca^{2+} waves. The scope of attenuation appeared to be a function of the buffer binding kinetics. The rate of Ca^{2+}-binding by CaBP was apparently too slow to influence Ca^{2+}-interaction between the closely situated IP_3 receptors in initiation sites, where the faster on-

rate buffer BAPTA exerted a significant effect. However, both CaBP and BAPTA had significant effects upon events which were more distal to the source of Ca^{2+} release, including effects between the more sparsely distributed IP_3 receptors involved in the propagation of Ca^{2+} waves, and global changes in Ca^{2+}. In view of the fact that Ca^{2+} waves and oscillations have been shown to modulate neuron development and gene expression, it is possible that the effects of CaBP may include influencing these processes.

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Dedication

To my parents, Gord Sr & Mary Rintoul.

Who taught me all the important stuff.

This thesis is a direct result of your love and support

Thanks Mum. Thanks Dad.

Chapter 1

General Introduction

Intracellular Calcium Homeostasis

Within the cells of all organisms, the level of the divalent cation calcium (Ca^{2+}) is maintained at a concentration greater than 10,000-fold lower than that of the extracellular environment. As early forms of life evolved utilizing a energy currency involving phosphates, the maintenance of a low intracellular calcium concentration ($[Ca^{2+}]_i$) was critical to cell survival, as high concentrations would result in phosphate precipitation. It seems likely that a corollary of this safety mechanism was the evolution of systems which utilized controlled calcium flux to transduce signals of internal and external origin. This change in $[Ca^{2+}]_i$ could be detected by calcium physically binding to target molecules or by the resultant charge migration. Thus the maintenance of a low $[Ca^{2+}]_i$ became necessary not only for cell survival but also to enable signal transduction; only against a precisely regulated background of resting $[Ca^{2+}]_i$ are cells able to sense and respond to influx or release of Ca^{2+} (Williams, 1990).

The low resting intracellular levels of Ca^{2+}, usually less than 100nM, are maintained through active processes which balance the slow leak of Ca^{2+} through the plasma membrane. These active processes result in Ca^{2+} being transported back across the plasma membrane or sequestered in intracellular organelles. Active extrusion of Ca^{2+} is largely handled by the high affinity, low capacity Ca^{2+}-ATPase in the plasma membrane while active sequestration is accomplished by similar ATP-hydrolysing pumps in the membrane of the endoplasmic (or sarcoplasmic) reticulum (ER / SR). When a high capacity for Ca^{2+} extrusion from the cytoplasm is required, active transport

of Ca^{2+} may also be carried out by the low affinity plasmalemal Na^{+}-Ca^{2+} exchanger (Fig.1).

Against this background of dynamic, regulated $[Ca^{2+}]_i$, the intracellular release or influx of Ca^{2+} from the external environment is able to mediate a myriad of cellular functions, either through 2nd messenger systems or effects on membrane potential. The physiological roles of the intracellular Ca^{2+} signal are very diverse, including gene transcription, contraction, cell division, neurotransmitter release (or secretion), metabolism, and learning and memory (for a recent, general review of functions see Berridge, 1998).

Influx of Extracellular Ca^{2+}

Changes in $[Ca^{2+}]_i$ can be achieved by the regulated movement of Ca^{2+} from the external environment into the cytoplasm through three classes of Ca^{2+} channels. The first are voltage gated channels which respond to membrane depolarization (reviewed by Armstrong and Hille, 1998). The second are mechanoreceptors, that allow influx of Ca^{2+} in response to cell membrane stretch or tension (Gotoh and Takahashi, 1999). The third are ligand-gated channels that are dependent upon the binding of other molecules for their activity including the N-methyl-D-aspartate (NMDA) subclass of glutamate receptors (reviewed by Hollmann and Heinemann, 1994).

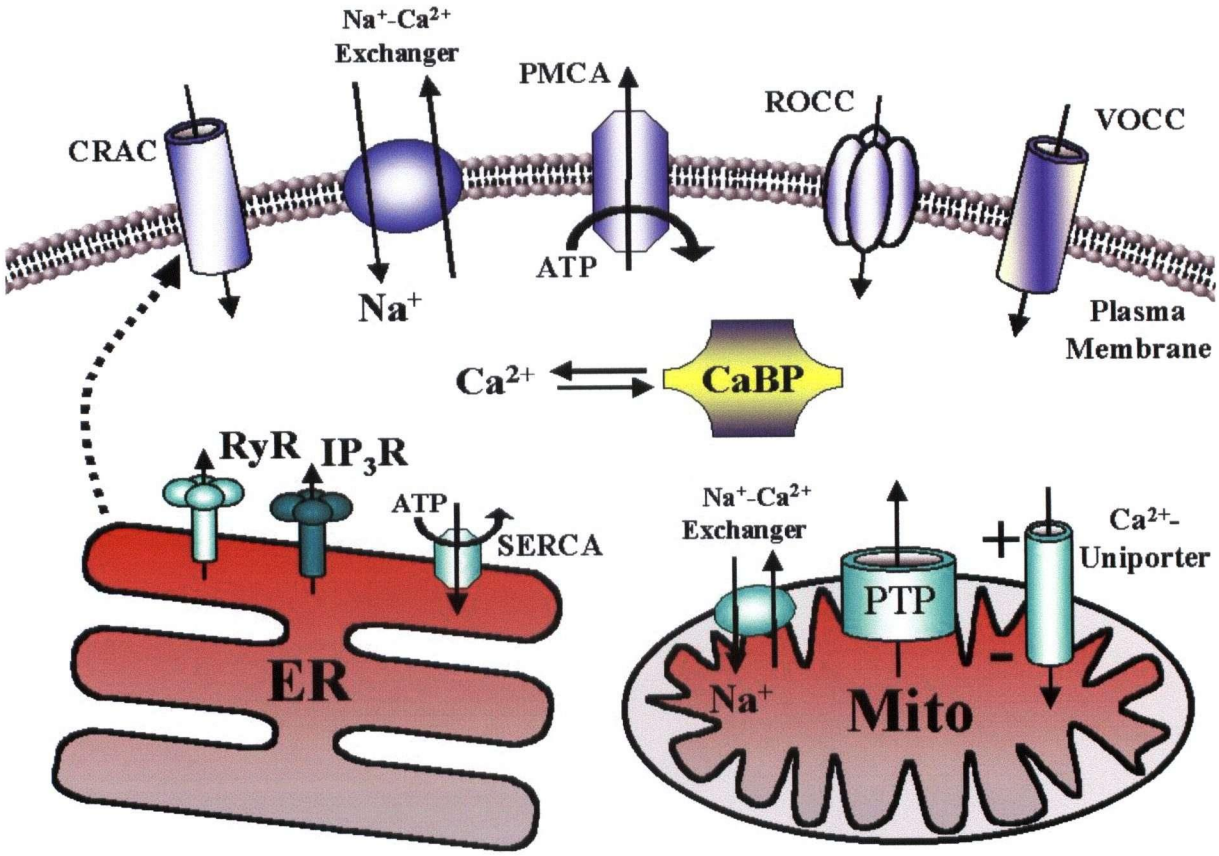


Figure 1. **Major routes of Ca^{2+} uptake and release involved in intracellular Ca^{2+} homeostasis.** All arrows indicate directional movement of Ca^{2+} unless otherwise indicated. ER - Endoplasmic Reticulum; Mito - Mitochondrion; CRAC - Calcium Release Activated Channel; PMCA - Plasma Membrane Ca^{2+}-ATPase; ROCC - Receptor-operated Ca^{2+} channel; VOCC - Voltage-operated Ca^{2+} channel; RyR - Ryanodine Receptor; IP_3R - IP_3-Receptor; SERCA - Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase; PTP - Mitochondrial Permeability Transition Pore

NMDA Receptors

Glutamate is the most prevalent excitatory neurotransmitter in the mammalian brain. A major class of glutamate receptors are those which function as ion channels. These ionotropic receptors have been classified according to their pharmacological and electrophysiological properties: α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors, kainate (KA) receptors, and NMDA receptors. The NMDA receptor, named for its selective activation by NMDA, has been the subject of intense scientific investigation, due largely to its suggested role in long-term potentiation (LTP) (Bliss and Collingridge, 1993) and Ca^{2+}-mediated cell death (reviewed by Choi, 1994).

NMDA receptors, like other ionotropic glutamate receptors, are permeable to Na^{+} and K^{+}. However, they differ from other receptor subtypes in this family in that they are also permeable to Ca^{2+} (MacDermott et al., 1986), are blocked by Mg^{2+} in a voltage dependent fashion (Nowak et al., 1984) and require glycine as a co-agonist (Johnson and Ascher, 1987). This channel therefore integrates chemical and electrical signals: in order for it to conduct ions it must bind glutamate and glycine and the Mg^{2+} block must be removed by membrane depolarization.

NMDA receptors consist of multiple subunits. Functional receptors all require NR1, a 97 kDa protein which, when expressed in Xenopus oocytes, conducts currents which are small relative to those measured in neurons, but nonetheless exhibits Mg^{2+}-dependent block and Ca^{2+} conductance (Moriyoshi et al., 1991). Fully functional NMDA receptors are likely hetero-oligomers of NR1 and one of four related subunits (NR2A-D). In experiments where NR1 and one of NR2A-2D proteins are co-expressed,

currents 5-60 times the amplitude of those measured in cells transfected with NR1 alone are recorded but no measurable currents are produced in experiments where NR2 subunits are expressed alone (Monyer et al., 1992).

Functional heterogeneity of NMDA receptors is achieved through differential expression of the NR2 subunits in the mammalian CNS. When combined with NR1, different NR2 subunits confer distinct pharmacological and ion channel properties to the NMDA receptor. The NR1 receptor is expressed widely in the brain (Petralia et al., 1994). However, expression of the various NR2 subunits varies with respect to the region of the CNS and stage of development (Watanabe et al., 1993, Monyer et al., 1992). This spatial and temporal variation in subunit expression provides for receptor diversity and therefore fine tuning of receptor activation and functional effects.

Functional roles have been attributed to the NMDAR based upon its ability to detect co-incidence of glutamate receptor binding and membrane depolarization. NMDAR participation in learning and memory, presumably through a mechanism related to LTP, has been extensively studied (for a current review see Malenka and Nicoll, 1999). The potential pathophysiological role of the NMDAR has also received much attention, being implicated in acute and degenerative neuronal cell death (Choi, 1992, 1995 ; Bonfoco et al., 1995).

Release of Ca^{2+} from Intracellular Pools

The ER of most cells constitutes a major intracellular store of Ca^{2+}. Uptake of Ca^{2+} from the cytoplasm occurs through active transport by sarco(endo) plasmic

reticulum ATPases (SERCA pumps) in the ER membrane. This family of ATPases consists of three members, SERCA1-3. SERCA 1 pumps are localized almost exclusively in fast-twitch skeletal muscle. SERCA 2 pumps exist in two isoforms as a result of alternative splicing: SERCA 2a is expressed in cardiac and smooth muscle and SERCA 2b appears to be the major form expressed in brain tissue. SERCA 3 is expressed in a variety of tissues, including the cerebellum (Wu et al, 1995). An understanding of the cellular role of SERCA pumps has been facilitated by the use of the tumor promoting agent thapsigargin which specifically blocks the action of SERCA pumps and causes depletion of ER Ca^{2+} stores (Thastrup et al., 1990).

Release of Ca^{2+} from the ER occurs through one of two related types of channels. In non-excitable cells, release of Ca^{2+} from the ER occurs through a receptor activated by the intracellular second messenger inositol (1,4,5) trisphosphate (IP_3). In excitable cells such as neurons, in addition to IP_3 receptors (IP_3R), the ER membrane contains channels which are directly activated by Ca^{2+}. These are called ryanodine receptors (RyR), due to their sensitivity to the plant alkaloid, ryanodine (reviewed by McPherson and Campbell,1993)

Mitochondria constitute the second major intracellular store of Ca^{2+}. Uptake of Ca^{2+} by mitochondria occurs via a Ca^{2+} uniporter, driven by the electrochemical gradient across the inner mitochondria membrane. Efflux of Ca^{2+} from mitochondria occurs through a Na^{+}/Ca^{2+} exchanger or the high conductance mitochondrial permeability transition (MPT) pore (Hunter and Haworth, 1979; Crompton et al., 1987). The MPT pore can be activated by high levels of intramitochondrial Ca^{2+} and has been suggested to play a role in apoptotic cell death (Lemasters et al., 1998).

IP_3 Mediated Calcium Signaling

HeLa cells, a prototypical non-excitable cell line, respond to application of histamine with IP_3 mediated intracellular Ca^{2+} release (Bootman and Berridge, 1996). Histamine initiates phospholipase C-mediated hydrolysis of the membrane phospholipid phophatidylinositol 4,5-bisphosphate (PIP_2) to DAG and IP_3. IP_3 diffuses rapidly in the cytosol and activates the IP_3-receptor (IP_3R) located on the surface of the ER. The activity of this channel is also modulated by cytosolic Ca^{2+}. This modulatory effect exhibits a bell shaped sensitivity, with a peak synergistic effect occurring at 200-300nM $[Ca^{2+}]_i$ (Bezprozvanny et al., 1991; Finch et al., 1991). This biphasic sensitivity of the receptor is thought to underlie the generation and propagation of Ca^{2+} waves and oscillations.

Oscillations of $[Ca^{2+}]_i$ have captured the attention of many researchers, as it has become apparent that oscillatory frequency can encode activation of various cellular processes such as gene expression (Berridge, 1997). As such, factors which can modulate calcium oscillations may play a critical role in defining how a particular cell type responds to external signals which induce intracellular calcium release.

Individual Ca^{2+} spikes within an oscillatory response are not simply global increases in $[Ca^{2+}]_i$. Each spike can be resolved temporally and spatially. Rises in cell Ca^{2+} begin in a defined region and spread throughout the cell. The defined "front" of this Ca^{2+} release has been described as a Ca^{2+} wave. It has been hypothesized that the basis of such waves involves a feed-forward mechanism, in which IP_3 acts upon IP_3 receptors

on the ER, releasing calcium which in turn acts synergistically with IP_3 to activate adjacent IP_3 receptors (Bootman et al., 1997).

In excitable cells, ryanodine receptors co-exist with IP_3 receptors on the surface of the ER. These receptors open in response to an increase in $[Ca^{2+}]_i$, and hence may play a part in the generation of Ca^{2+} waves and oscillations in these tissues. Therefore, in either excitable or non-excitable tissue, a major determinant of $[Ca^{2+}]_i$ dynamics may involve Ca^{2+} feedback or feed-forward mechanisms. In this context, it is probable that any factor which may compete with IP_3 or ryanodine receptors for calcium, or which may influence the diffusion of calcium, may modulate the generation and/or propagation of calcium waves and oscillations.

Calbindin-D28K

Another possible fate of cytoplasmic Ca^{2+} is that of binding to intracellular Ca^{2+}-binding proteins. Currently these proteins are classified according to their putative function: either "trigger" or "buffer" (Baimbridge et al., 1992). Trigger proteins such as the ubiquitous calmodulin are components of second messenger pathways mediating many cellular functions. The function of buffer proteins is to bind excess Ca^{2+} as $[Ca^{2+}]_i$ increases, although it is possible that these proteins have as yet undiscovered targets. One member of this protein family is calbindin-D28K (CaBP).

Previously known as vitamin D-dependent calcium binding protein, CaBP is a 28kD intracellular calcium binding protein (Wasserman and Taylor, 1966). Examination

of the amino acid sequence of CaBP reveals six regions which encode the consensus sequence for the helix-loop helix structure of the "EF-hand" motif responsible for high-affinity Ca^{2+}-binding (Parmentier et al., 1987). However, under physiological conditions only four of these Ca^{2+} -binding sites appear to be functional (K_d =513 nM) (I. Mody, Personal communication; Bredderman and Wasserman, 1974; Cheung et al., 1993) (Table 1). In the calcium binding protein superfamily, trigger proteins such as troponin C or calmodulin are components of second messenger cascades, undergoing conformational changes upon binding of Ca^{2+} and subsequently modulating the activity of target molecules. These proteins are not uniformly distributed within cells but are often concentrated in specific regions such as the post-synaptic density where they bind to intracellular cytoskeletal or contractile elements. CaBP is generally considered to be a member of the "buffer" category of calcium binding proteins along with parvalbumin and calretinin. These proteins are distinguished by the fact that they appear to be uniformly distributed within the cytoplasm of cells that express them (Baimbridge et al., 1992). Although the hypothesized role of CaBP is to act as a buffer of intracellular Ca^{2+}, it has yet to be demonstrated that Ca^{2+}-buffering is its exclusive role. Indeed, recent structural (Berggard et al., 2000) and experimental (Bellido et al., 2000) observations lend support to the idea that CaBP may not only function as an intracellular Ca^{2+} buffer, but may interact with target proteins in the cell.

CaBP was originally isolated from chick epithelial tissue by Wasserman and Taylor (1966), and subsequently shown to be found in many other Ca^{2+}-transporting epithelia, including the distal tubule of the kidney and the chicken oviduct. In Ca^{2+} transporting cells, the synthesis of CaBP is regulated by the active metabolite of vitamin

	k_{on} ($x10^6$ $M^{-1}s^{-1}$)	k_{off} (s^{-1})	Kd (nM)
BAPTA	600 [1, 2]	300 [3]	500 [1]
EGTA	1.5 [1]	0.45 [4]	860 (pH 7.0) [1] 220 (pH 7.3) [1]
FLUO-3	920 [5]	424 [5]	462 [5]
FURA-2	760 [5]	109 [5]	144 [5]
CaBP-9k	20 [6]	8.6 [6]	430 [6]
CaBP-28K	77 [7]	39.5 [7]	513 human [7] 300 chick [8]

Table 1. Constants (Kd, k_{on} and k_{off}) of Ca^{2+} chelators, Ca^{2+}-indicators and Ca^{2+} binding proteins. Constants were determined at 22°C unless otherwise indicated. References: (1) Adler et al., 1991. Reported values were measured under physiological conditions. (2) Tsien, 1980. (3) Pethig et al., 1989. (4) Harafuji and Ogawa, 1980. (5) Lattanzio and Bartschat, 1991. (6) Feher et al., 1992. (7) I. Mody and U.V. Nägerl, UCLA, Personal Communication .Unpublished results of experiments employing recombinant protein generated as described in materials and methods. (8) Cheung et al., 1993.

D, 1,25-dihydroxy-cholecalciferol in a typical steroid-like manner. Expression of CaBP is not limited to Ca^{2+}-transporting tissues. It has been detected in many vertebrate tissues with notable exceptions being striated muscle and mammalian intestinal epithelium (Wasserman and Fullmer, 1982). In vertebrates, CaBP is found in both central and peripheral nervous systems. CaBP expression in the nervous system differs from expression in Ca^{2+}-transporting tissues in that it is not dependent on vitamin D (Baimbridge and Parkes, 1981; Varghese et al., 1988).

The concentration of CaBP within rat cerebellar Purkinje cells has been estimated to be about 0.1-0.2 mM, and since each molecule can bind up to four Ca^{2+}, the total Ca^{2+}-buffering capacity is therefore in the order of 0.4-0.8 mmol/litre cytoplasm (Baimbridge et al., 1982). This extraordinarily large buffering capacity is similar to that found in the absorptive cells of the chick gut (Feher et al., 1992), and a similar concentration has been reported for rat cerebellar Purkinje cells using a quantitative immunohistochemical procedure (Davenport et al., 1990) and a fura-2 titration method (Fierro and Llano,1996). In the absence of evidence for a "trigger" function it has been speculated that this protein may form a major intraneuronal Ca^{2+} buffering system, (Baimbridge and Parkes, 1981; Baimbridge et al., 1982; Jande et al., 1981; Mody et al., 1987).

Distribution of CaBP in the CNS and Electrophysiological Correlates

Within the nervous system CaBP is found in distinct neuronal populations such as cerebellar Purkinje cells and the principal cells of the dentate gyrus and CA1/CA2 sub-fields of the hippocampal formation (Baimbridge and Miller,1982; Buchan and Baimbridge,1988; Celio,1990; Jande et al., 1981). The distribution studies have described the presence of CaBP in a variety of neurons with different functions, neurotransmitter content or receptor profiles. For example, CaBP is found in major projecting pathways that are either inhibitory (Purkinje cells) or excitatory (hippocampal dentate granule and CA1 pyramidal neurons), as well as local circuit inhibitory interneurons in the cortex. CaBP is found in some, but not all neurons with a high density of NMDA receptors or IP_3 receptors, and examples could be given of CaBP associated with almost all of the known classical neurotransmitters (Baimbridge et al., 1992). The intra-neuronal distribution of CaBP appears to be largely cytosolic, with an immunohistochemical staining pattern that is even in intensity throughout the cell volume as observed with light microscopy, and no evidence of association with specific membranes or organelles in Purkinje neurons observed at the EM level (Pasteels et al., 1986).
Kawaguchi and Kubota (1993) examined the electrophysiological properties of rat frontal cortex neurons and correlated their response with the presence of either CaBP or parvalbumin (PV). PV was present in neurons which showed fast-spiking characteristics in response to either intracellular current injection or synaptic stimulation. This result was consistent with findings in rat hippocampal CA1 interneurons (Kawaguchi et al., 1987) and a general association of PV with neurons that are functionally more active (Celio, 1990). CaBP-positive neurons had low threshold spikes and a distinct morphology compared with the PV-positive

neurons, but both CaBP-positive and -negative neurons in the rat striatum show similar low threshold spikes (Kawaguchi et al., 1987). In an interesting report, Li et al (1995) determined that introduction of CaBP into rat supraoptic neurons using patch clamp technology, suppressed Ca^{2+}-dependent depolarizing afterpotentials and changed activity from phasic to continuous firing. However, Baimbridge et al (1991) failed to show an association between the presence or absence of CaBP and either non-accommodating or bursting responses to current injection in rat CA1 pyramidal neurons, and others failed to show any relationship between CaBP and high threshold Ca^{2+} conductances in guinea-pig lateral septal neurons (Doutrelant et al, 1993).

Does CaBP have a Neuroprotective Role in the CNS ?

Mattson et al (1991) reported a correlation between the presence of CaBP and a relative sparing of cultured hippocampal neurons to excitotoxic levels of glutamate. However, since CaBP-positive neurons represent a distinct phenotype, it is certainly conceivable that other factors may have contributed to neuron sparing. For example, there may be reduced numbers of EAA receptors or voltage operated Ca^{2+}-channels (VOCCs) which could result in a reduced Ca^{2+} entry during exposure to excitotoxins. These result were not supported by Möckel and Fisher (1994) who found no significant correlation between the presence of CaBP and sensitivity to excitoxicity in similar cultures.

The effects of artificial Ca^{2+} buffers on changes in EAA and high K^+-evoked increases in $[Ca^{2+}]_i$ in cultured hippocampal neurons and their effects upon susceptibility to

excitotoxicity have been examined (Abdel-Hamid and Baimbridge, 1997). Preloading neurons with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) resulted in an effective buffering of increases in $[Ca^{2+}]_i$ induced by transient (<25 sec) exposure to NMDA or 50 mM K^+ as was evident by the reduction in the rate of rise and the peak $[Ca^{2+}]_i$ response, together with a significant prolongation of the time to recover to prior resting $[Ca^{2+}]_i$ levels. In addition, an *enhanced* susceptibility to excitotoxicity in BAPTA-loaded neurons has been reported (Dubinsky,1993; Abdel-Hamid and Baimbridge, 1997). More recently, it has been shown that neurons naturally expressing CaBP have larger $[Ca^{2+}]_i$ responses, but, like BAPTA, these responses are prolonged and excitotoxicity is enhanced (Abdel-Hamid et al, Unpublished Results). As with Mattson et al (1991) the problem of the correlative nature of the data arises, and alternative explanations are possible.

Evidence supporting a neuroprotective role of CaBP has been reported by Goodman et al (1993) in which only those later-developing granule cells close to the hilus (which are devoid of CaBP-immunoreactivity; Baimbridge, 1992) were vulnerable to anoxia-induced cell death in 7 day old rat pups. In a similar model it has been shown that the non-CaBP containing neurons in the dorso-lateral quadrant of the striatum are more sensitive to anoxia-induced cell death than the CaBP-positive neurons in the ventro-medial quadrant (Burke and Baimbridge, 1993). Additionally Sloviter (1989), using a perforant path stimulation model of epilepsy, has suggested that hilar neurons devoid of either CaBP or PV immunoreactivity, were selectively vulnerable, an argument elegantly supported by Scharfman and Schwartzkroin (1989) who showed that these vulnerable neurons could be protected by the presence of BAPTA. A further model of cell death that has supported a neuroprotective role of CaBP, and one which has been reproduced in a number of laboratories, is the selective

destruction of dopamine neurons in the substantia nigra pars compacta (SNc) following treatment with the neurotoxic agent, 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (German et al., 1992; Iacopino et al., 1992; Lavoie et al., 1991). The essential finding is that the dorsal tier of dopamine neurons in the SNc which contain CaBP (Gerfen et al., 1985, 1987) are relatively spared from cell death induced by MPTP treatment. This data is also entirely correlative and there are other possible explanations. For example, the CaBP containing dorsal tier of dopaminergic neurons in SNc have lower levels of the dopamine transporter which is required for MPTP uptake (Sanghera, 1994).

Evidence against a neuroprotective role of CaBP can be found by a careful examination of the effects of global forebrain ischemia in the rat. This results in the complete destruction of the CA1 pyramidal neurons in the hippocampal formation, regardless of whether or not they contain CaBP (Freund et al., 1990; Mudrick and Baimbridge, 1989). CA3 neurons are resistant to ischemia-induced cell death and yet these neurons contain neither CaBP, PV or Calretinin, another neuron specific calcium binding protein with a high homology with CaBP but distinct neuronal distribution (Rogers, 1987). This lack of correlation between calcium-binding protein content and vulnerability to ischemia is in fact not just restricted to the hippocampus but extends to the neocortex, thalamus and striatum (Freund et al., 1990). Further evidence against a neuroprotective role has been obtained from studies of the effects of ischemia on CaBP "knockout" mice (CB -/-) in which *less* damage occurred in the CA1 region when compared to control (CB +/+) littermates with their normal complement of CaBP (Klapstein et al., 1998).

Studies in Human Disease

CaBP has been detected immunohistochemically in distinct populations of human neurons, the loss of which has been clearly identified with a particular disease. For example CaBP is present in the cholinergic neurons of the nucleus basalis of Meynert (Celio and Norman, 1985), and some of the cortical neurons which are lost in Alzheimer's disease (Ichimiya et al., 1988; Jande et al., 1981). Similarly, CaBP is present in a very large percentage of the striatonigral projecting neurons (Gerfen et al., 1985, 1987) which are lost in Huntington's disease (Seto-Oshima et al., 1988). Seto-Oshima et al (1988) have proposed that the normal presence of CaBP in the neostriatal neurons, which receive a major excitatory input from the cortex, may serve to prevent excitotoxic effects and that the selective loss of these neurons in Huntington's disease may reflect a failure of the normal Ca^{2+}-buffering capability. In contrast, there is a relative sparing of the CaBP containing neurons in the dorsal tier of dopamine neurons in the human SNc when examined in post-mortem samples from Parkinson's disease patients (Yamada et al., 1990). Further aspects of this topic have been reviewed (Heizmann and Braun, 1992).

Functional Studies of CaBP in the CNS and Model Cell Lines

A specific reduction of CaBP has been reported in the dentate granule cells of the rat hippocampal formation following kindling (Baimbridge and Miller, 1984; Baimbridge et al., 1985; Mody et al., 1987) and a similar loss of CaBP in dentate granule cells from human

temporal lobe epilepsy has been recently reported (Magloczky et al., 1997). Using the kindling model, Köhr et al (1991) have associated this loss of CaBP with a reduced Ca^{2+} entry through voltage-operated Ca^{2+} channels. They interpreted this data as indicating that the loss of CaBP allows for a more efficient Ca^{2+} dependent inactivation of the Ca^{2+} channels, and this was supported by the ability of the presence of BAPTA in the recording electrode to reverse this effect. It should be noted that the implication of this result is that the presence of a Ca^{2+}-buffer will increase the net flux of Ca^{2+} into a neuron, consistent with our own observations (Abdel-Hamid et al, Unpublished Results), and the effects of BAPTA upon excitotoxicity reported by Abdel-Hamid and Baimbridge (1997). However, Chard et al (1993) did not observe any effect of CaBP on Ca^{2+} channel inactivation following its direct injection into rat dorsal root ganglion neurons. They did find an 8-fold decrease in the rate of rise in $[Ca^{2+}]_i$, a reduction in the peak $[Ca^{2+}]_i$ and an altered kinetics of decay of $[Ca^{2+}]_i$ to a single slow component, following a brief depolarization. Thus they concluded that CaBP could act as a Ca^{2+} buffer but that this effect had no influence on Ca^{2+} channel kinetics.

Transfection of the gene for CaBP has been achieved in a number of different cells. In GH3 cells CaBP overexpression was associated with a reduced Ca^{2+} entry through VOCCs and a reduction in $[Ca^{2+}]_i$ transients evoked by depolarization (Lledo et al., 1992). In lymphocytes, overexpression of CaBP was associated with an increased survival in the presence of the Ca^{2+} ionophore A23187 and a decrease in the apoptotic effects of dexamethasone and forskolin (Dowd et al., 1992). In cultured rat neurons, transfection of CaBP using adenovirus was associated with a loss of post-tetanic potentiation (Chard et al., 1995). In motoneuron hybrid cells, transfection of CaBP with a retrovirus prevented amyotrophic lateral sclerosis IgG-mediated cytotoxicity (Ho et al., 1996). Despite these

interesting findings it is clear that the results of these transfection studies, and those using other means of experimentally manipulating levels of CaBP, have not resolved the function of CaBP. Indeed, in some case, the data is entirely conflicting (Köhr et al.,1991; Lledo et al., 1992).

Excitotoxicity

The concept of "excitotoxicity" was introduced by Olney et al (1971) to describe the deleterious effects of excessive stimulation of neurons with EAA. The essential role of Ca^{2+} in cell death was suggested by Schanne et al (1979). Their "calcium hypothesis" of cell injury proposed that a massive influx of Ca^{2+} mediates, or initiates a "final common pathway" to cell death. Influx of Ca^{2+} through NMDAR has been implicated in numerous neuropathologies, largely due to the apparent ability of NMDAR antagonists to ameliorate glutamate mediated neurotoxicity (reviewed by Choi, 1988).

The central role of Ca^{2+} in excitotoxicity has been underscored in more recent reports which have attempted to correlate induced increases in $[Ca^{2+}]_i$ and subsequent cell death. Studies employing the fluorescent indicator fura-2 reported no significant correlation between the magnitude of NMDAR mediated $[Ca^{2+}]_i$ increase and cell death (Tymiyanski et al., 1993a; Dubinski and Rothman, 1991). On the basis of these results, it was hypothesized that the route of Ca^{2+} entry may be a more important determinant of cell survival following Ca^{2+} influx (Tymianski et al., 1993a). This hypothesis has received support through claims of "privileged access" of Ca^{2+} from NMDAR to

mitochondria, the reported mediators of apoptotic cell death (Peng and Greenamyre, 1998). However, studies which examined $^{45}Ca^{2+}$ influx in response to NMDAR activation did report a correlation between amount of Ca^{2+} influx and cell death (Eimerl and Schramm, 1994; Hartley et al., 1993; Lu et al., 1996). This apparent discrepancy may be explained by recent reports which have examined the accuracy of high affinity calcium indicators (such as fura-2) in $[Ca^{2+}]_i$ determination. Hyrc et al (1997) demonstrated that fura-2 selectively underestimates the $[Ca^{2+}]_i$ associated with influx through NMDAR. Stout and Reynolds (1999) confirmed these findings and reported that when low affinity calcium indicators were employed, there was indeed a correlation between $[Ca^{2+}]_i$ and cytotoxicity.

Interestingly, transfection and activation of the cloned subunits of the NMDAR into non-neuronal cell types can produce "acquired excitoxicity": co-expression of the NR1 and NR2A/B subunits has been shown to mediate increases in $[Ca^{2+}]_i$ (Grant et al., 1997) and leads to excitotoxic cell death (Cik et al., 1993; Anegawa et al., 1995; Boekman and Aizenman, 1996; Raymond et al., 1996).

Rationale

On the basis of its known Ca^{2+}-binding properties, and in view of the lack of evidence to support a modulatory role for this protein, it has been speculated that CaBP functions primarily as an intracellular Ca^{2+}-buffer that may serve to protect neurons from the potentially harmful effects of large and/or prolonged increases in $[Ca^{2+}]_i$. However, evidence in the literature provides support both for and against such a function, possibly in part due the many different experimental approaches that have sought to elucidate such a function, both *in vivo* and in *vivo*. The objective of the present studies was to utilize simple cellular systems to examine both the proposed Ca^{2+}-buffering and protective functions of CaBP. The use of stably transfected cell lines ensured that the only variable altered between control and experimental cells would be the expression of CaBP. In addition, much of the interest in the function of CaBP has been focussed on its potential role under pathophysiological situations with little attention being placed upon its function(s) under physiological conditions. Therefore, a further objective of the present studies was to investigate the influence of CaBP upon intracellular Ca^{2+}-signalling events such as Ca^{2+} oscillations and waves.

Hypotheses

1) Transfection of CaBP into non-neuronal cell lines will enhance the cell's endogenous Ca^{2+}-buffering capacity.

2) Increased Ca^{2+}-buffering in HEK 293 cells by transfected CaBP will enhance cell survival following activation of recombinant NMDA receptors.

3) Transfected CaBP will modulate the temporal and spatial characteristics of IP_3-induced Ca^{2+} release from intracellular stores in HeLa cells, decreasing the frequency of Ca^{2+} oscillations and attenuating the velocity of Ca^{2+} waves.

Chapter 2

Materials and Methods

Cloning and Expression of the Human Recombinant CaBP

The molecular biology techniques employed in this study were based on those described in Molecular Cloning, A Laboratory Manual by Sambrook et al., 1989 and Current Protocols in Molecular Biology, edited by Frederick M. Ausubel, 1987. Additional details are provided where modifications were made to these procedures.

Polymerase Chain Reaction

The human CaBP gene was cloned from a human fetal brain cDNA library (Promega) via polymerase chain rection (PCR) amplification. Primers were designed to incorporate restriction sites which would facilitate cloning of the gene into the expression vectors (Fig. 2A). Design and analysis of oligonucleotide primers was carried out utilizing Oligo 4.0 Primer Analysis Software (Wojciech Rychlik). Restriction analysis of all DNA sequences was carried out using the computer program PC/Gene (Intelligenetics Inc).

Primers were synthesized on an Applied Biosystems 380A DNA synthesizer. Oligonucleotides were eluted from the synthesis column with 2 mL of 8 M NH_4OH. Following overnight incubation in a 55°C water bath, the ammonium hydroxide was removed by evaporation in a Sorvall Speed Vac Centrifuge for 4 hours. The oligonucleotide pellet was resuspended in 200 μL of distilled water and quantified by

measuring the absorbance at 260 nm. (1unit A260 = 33 μg/mL of single stranded DNA).

The optimal quantity of the amplified cDNA library template was determined empirically by diluting 1 to 10,000 fold. PCR reactions consisted of various dilutions of the cDNA library template DNA, 100 ng of each oligonucleotide, 0.05 mM dNTPs (Pharmacia), 1X Buffer E (67 mM Tris Base pH 9.01, 1.5 mM magnesium sulfate, 166 mM ammonium sulfate, 10 mM β-mercaptoethanol), and 5 units of *T. aquaticus* polymerase in a total volume of 50 μL. Reaction mixtures were overlayed with 3 drops of mineral oil, then cycled on a Perken Elmer thermocycler as follows: 1 minute at 94 °C, 1 minute at 50 °C, and 1 minute at 72 °C, for 25 cycles. Amplified fragments were separated by electrophoresis in a 1% agarose gel in the presence of 1μg/mL ethidium bromide. DNA fragments were visualized by ultraviolet light illumination and photographed with a Polaroid camera.

An ~800bp band, corresponding to the coding region of CaBP was excised from the agarose gel and eluted using a "Geneclean" kit (Bio 101) according to the manufactures instructions (Fig. 2B). Eluted fragments were cut with the restriction enzymes EcoRV and XbaI (Life Technologies) and ligated to corresponding restriction sites in the cloning vector pBluescript II (Stratagene).

A

H5E1:

5'- ATC **GAT ATC** ATG GCA GAA TCC CAC CTG CAG-3'

H3X1:

5'-ATC **TCT AGA** GTG GTT GCG GCC ACC AAC TCT A-3'

B

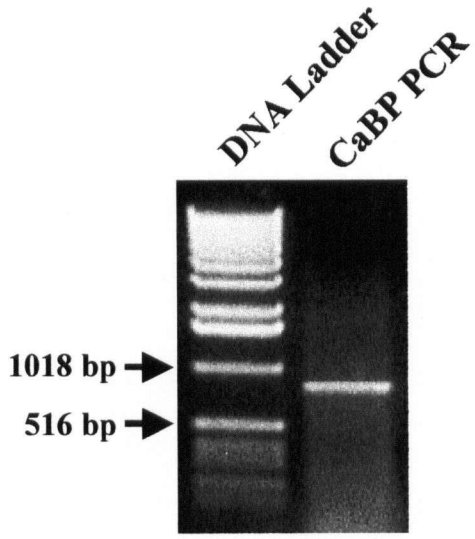


Figure 2. PCR of Human CaBP cDNA. (A). Primers employed to amplify human CaBP cDNA. H5E1 incorporates the *EcoRV* restriction site (bold) along with the start codon (underlined) and the 5' end of the CaBP gene. H3X1 targets the stop codon (underlined), and a segment of the 3'-untranslated region. It incorporates a recognition sequence for the restriction endonuclease *Xba*1 (bold). (B) Ethidium bromide stained agarose gel of DNA electrophoresis of human CaBP gene PCR amplification product.

DNA Sequence Analysis

DNA sequence analysis was carried out using a T7-Sequencing Kit (Pharmacia) as per the manufacturer's instructions, with the following modifications. DNA template (10 μg), combined with 100 ng of a sequencing primer was denatured with 0.2 N sodium hydroxide in a volume of 20 μL in a boiling water bath for 2 minutes, followed by snap cooling on ice. The DNA was subsequently precipitated with 0.1 volumes of 3 M sodium acetate pH 5.6, and 2.5 volumes of 95% ethanol. Precipitated DNA was collected by centrifugation in an Eppendorf micro-centrifuge for 15 minutes at 14,000 rpm. The DNA pellet was then resuspended in 10 μL of 1X Annealing Buffer (40 mM Tris base pH 7.5, 20 mM magnesium chloride, 50 mM sodium chloride). To the DNA template and annealed primer solution was added 1 μL of 0.1 M di-thiothreitol, 2μL 1X labelling mix, 5 μCi α^{35}S-dATP, 2 μL of a 1:8 dilution of *Sequenase* enzyme, and this mixture was left to incubate at room temperature for 2 minutes. In a 60 well micro-titre plate (Nunc), 2.5 μL of 80 μM dideoxyguanosine, dideoxyadenosine, dideoxythymidine, and dideoxycytidine in 50 mM sodium chloride were added to separate wells and warmed to 37°C on a heating block. The above "labelling reaction" (3.5 μL) was added to each of the wells containing the respective dideoxy-nucleotides and incubated at 37 °C for 5 minutes. A termination solution (4μL, 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF) was then added to terminate the reaction. Samples were boiled in a boiling water bath for 2 minutes, then immediately transferred to ice.

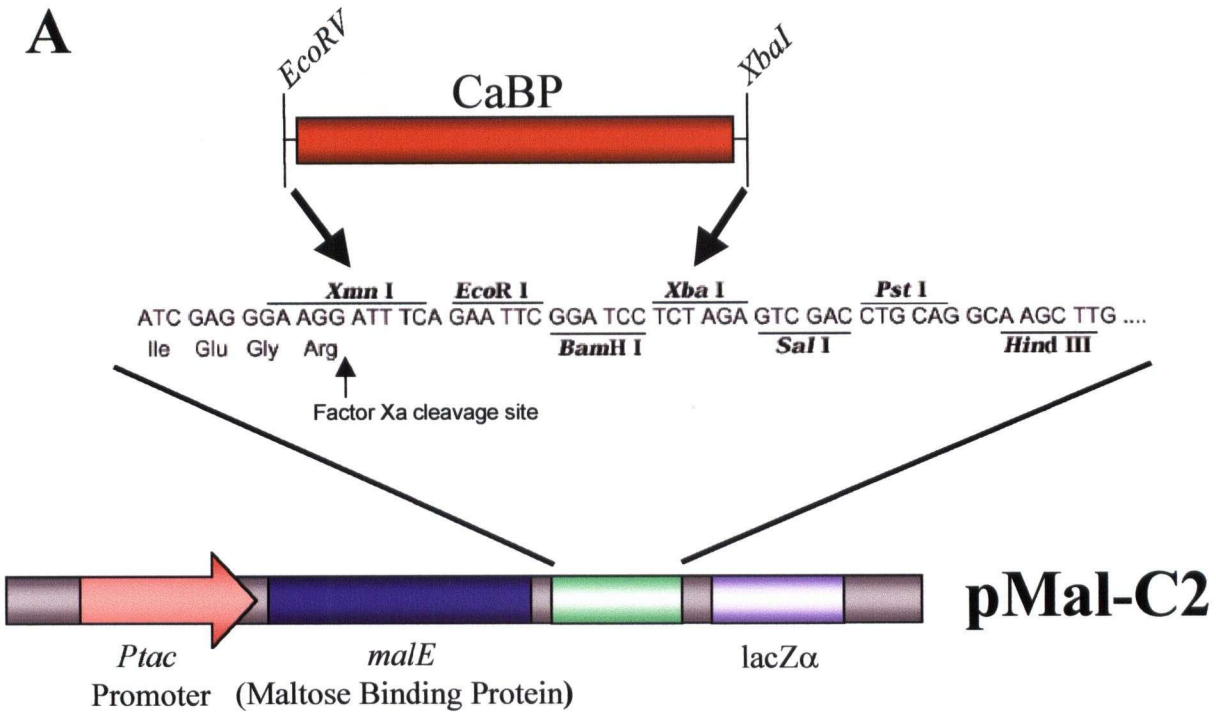
Samples were subjected to electrophoresis in a 10% acrylamide, 8M urea, 1X TBE (0.89 M Tris base, 0.89 M boric acid, 25 mM EDTA; pH 8.0) gel on a Model S2 sequencing apparatus (Life Technologies) for up to 5 hours. Following electrophoresis of the sequencing reactions, gels were dried under vacuum with a Bio-Rad gel drier at 80°C for 1 hour and the labeled nucleic acid fragments were visualized by autoradiography using film from Island Scientific.The CaBP DNA sequence was subsequently verified by automated DNA sequencing (Nucleic Acid and Protein Sequencing Unit, UBC).

Protein Expression

The coding region of CaBP was inserted, in frame, into the bacterial expression vector pMAL-C2 (New England Biolabs) to allow the expression of large quantities of recombinant protein. The entire CaBP coding region was excised from pBluscript II with the restriction endonucleases EcoRV and XbaI, and inserted into the XmnI and XbaI restriction sites of the pMal-C2 vector. The DH5-α strain of *E.coli* was transformed with this construct and production of the full-length fusion protein was confirmed by SDS-PAGE and western blot detection of CaBP. The resultant protein from this expression system is a fusion protein of CaBP and Maltose Binding Protein (MBP), separated by a Factor Xa protease recognition site. Protein expression and purification was carried out according to the manufacturer's instructions. Briefly, following confirmation of correct insertion of the CaBP sequence into the pMAL vector

by restriction analysis, the expression vector was transformed into *E.coli* and grown at 37°C in 1 liter of LB broth to an OD of 0.5 (measured at 600 nm). The expression of the MBP-CaBP fusion protein was induced by addition of 2.5 mM IPTG, and the culture was allowed to incubate for a further two hours at 37°C with constant agitation. Bacteria were collected by centrifugation, washed, and resuspended in a Tris buffer (20 mM Tris pH 7.4, 200 mM NaCl, 1 mM EDTA). Lysis of the resuspended bacteria was carried out by sonication at 4°C. Cell membranes and denatured proteins were removed by centrifugation. The crude soluble protein fraction was then passed through a maltose column which separated the fusion protein from the soluble bacterial lysate. The fusion protein was eluted from the column with 10 mM maltose in Tris buffer. The fusion protein was cleaved by digestion with bovine protease Factor Xa (0.1 % w/v of total fusion protein). The cleaved protein mixture was passed through the maltose column once again to remove the MBP. The CaBP protein was concentrated with a mini-Amicon concentrator and a 10,000 kDa cutoff filter.

The correct size of the purified protein was confirmed by SDS-PAGE. Western blot analysis of the purified protein revealed that the protein was recognized by an anti-chick CaBP antibody, which has been shown previously to cross react with human CaBP (Fig. 3). Further characterization of the recombinant CaBP was performed in the lab of Dr. Les Burtnick (Department of Chemistry, UBC). It was confirmed that the recombinant CaBP binds Ca^{2+}, and undergoes a conformational change upon binding Ca^{2+} similar to that observed for avian CaBP (Gross et al., 1987) (Fig. 4). Mass Spectroscopy of the purified protein, performed by Dr. Michael Murphy (Department of Microbiology, UBC), yielded an interesting result. A protein of double the molecular



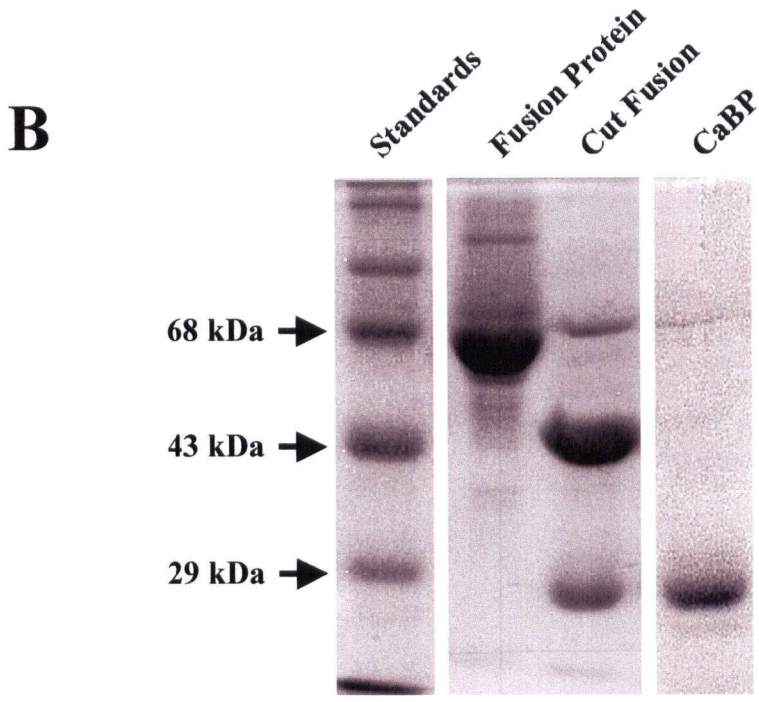


Figure 3. Purification of recombinant human CaBP. A) pMal Expression vector construct. B) SDS PAGE of the stages of purification. Shown are the fusion protein from the bacterial lysate (Fusion Protein), the result of cleavage of the fusion protein with Factor Xa (Cut Fusion) and the purified CaBP protein.

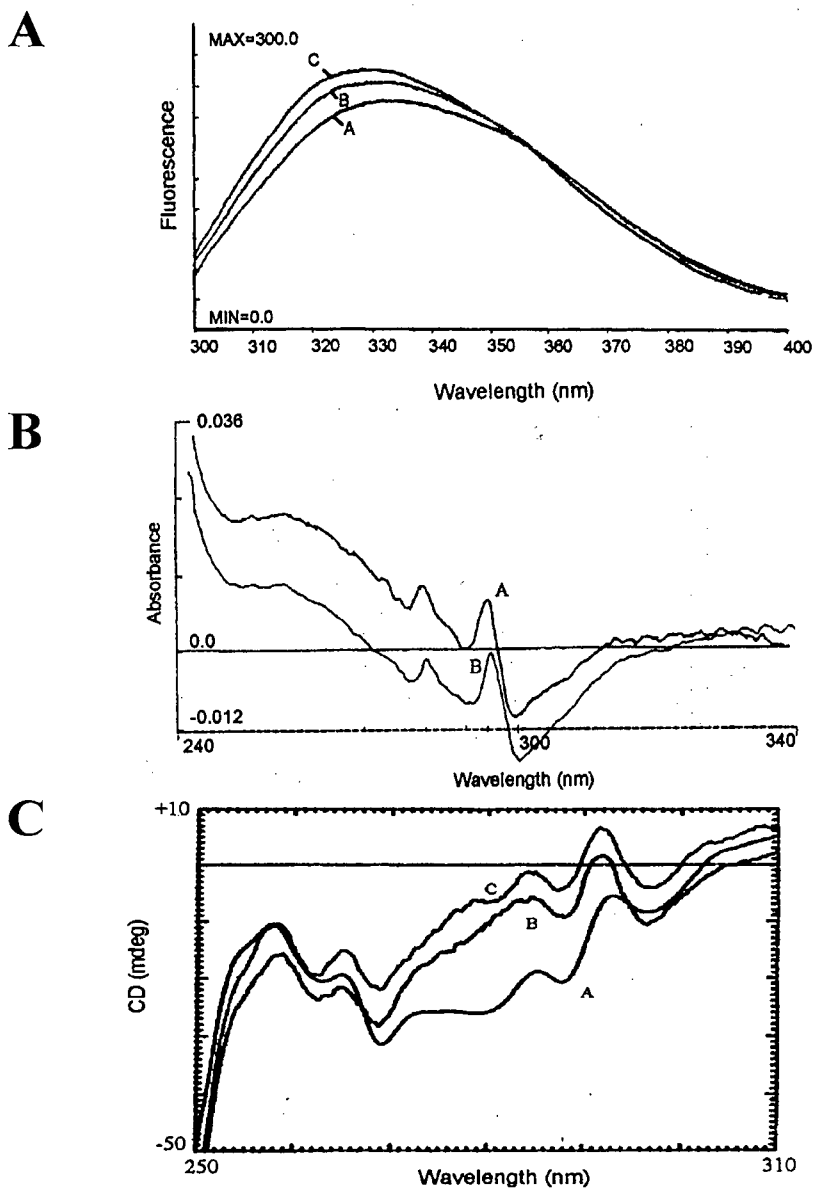


Figure 4. Spectrophotometric studies of recombinant human CaBP demonstrating a conformational change upon binding Ca^{2+}. **A.** Fluorescence emission spectra for human CaBP in the presence and absence of Ca^{2+}; (A) 0 mM Ca^{2+} (B) 0.2 mM Ca^{2+} (C) 0.6 mM Ca^{2+}. The observed changes in fluorescence suggest a conformational change upon Ca^{2+} binding, causing a tryptophan residue to move into a more hydrophobic region. **B.** Difference spectrum of Ca^{2+} bound recombinant human CaBP. The reference cell contained Calbindin in buffer with no Ca^{2+}. The trace depicts the result of adding (A) 1 mM and (B) 1.8 mM Ca^{2+}. The results indicate that Ca^{2+} causes a conformational change, moving aromatic chromophores to a more hydrophobic environment. **C.** Near UV circular dichroism spectra of recombinant human calbindin in (A) 0 mM Ca^{2+} ,(B) 1.8 mM Ca^{2+} and (C) 2.6 mM Ca^{2+}. The changes depicted in the trace are a result of changing environments of aromatic chromophores incurred by protein conformational changes (Spectrophotometric studies carried out by Seung Min Cha in the Lab of Dr. Les Burtnick, Department of Chemistry, UBC).

weight (minus 2 hydrogens) calculated from the amino acid sequence (30,025 kDa) was detected. This apparent dimerization was confirmed by SDS-PAGE, and was usually observed in the absence of DTT or other reducing agents, suggesting that dimerization was the result of a disulfide linkage. In addition, the degree of dimerization increased significantly if solutions of CaBP were concentrated above 1mg/mL. Care was therefore taken to limit all CaBP containing solutions to less than 1mg/mL. Disulfide-mediated dimerization of CaBP has been reported by Berggard et al, (2000) who speculate that this is not a physiologically relevant phenomenon, owing to the reducing environment of the cell cytosol.

Generation and Characterization of Stable Cell Lines

Mammalian Cell Culture

HEK 293 (Quantum Biotechnologies) and HeLa (A gift from Dr. Alison Buchan, Department of Physiology, UBC) cultures were maintained in Dulbecco's minimal essential medium (DMEM) containing 5% fetal bovine serum (Life Technologies) at 37 °C in an atmosphere of 95% air, 5% CO_2.

Stable Expression of CaBP

Expression vector DNA for transfection was prepared using a Qiagen Maxi-prep kit according to the manufacturer's instructions. The human CaBP gene was inserted into the pCDNA3 expression vector (Invitrogen; Carlsbad, CA), which contains the neomycin (G418) resistance gene. HEK 293 and HeLa cultures were transfected using standard calcium phosphate precipitation transfection techniques with 10μg of expression vector DNA. Briefly, DNA was diluted in a 40mM $CaCl_2$ solution in a total volume of 250 μL. This solution was added drop-wise to 250 μL of 2xHBS. After a 1 minute incubation at room temperature this transfection solution was added to a 10 cm plate of cells which had been plated the previous day at 10^6 cells per plate. Following overnight incubation, cells were washed twice with fresh media, then split between two 10 cm plates and incubated in the presence of G418 (Life Technologies; 1 mg/mL G418 for HEK 293 cells; 0.5 mg/mL for HeLa cells). Control cell lines were generated by stably transfecting with the empty pCDNA3 vector. Individual colonies of cells resistant to G418 were isolated and amplified. Expression (or absence, in control cultures) of CaBP was verified by immunohistochemical staining of cultures as previously described (Buchan and Baimbridge, 1988) and western blot analysis (Fig. 5). One HEK 293 clone (HEK-CB12) was selected for these studies on the basis of its relatively high intensity of staining observed by immunohistochemistry (IHC). A control cell line (HEK-p3) was also generated that was stably transfected with the pCDNA3 vector alone. In all HeLa cell lines isolated, expression of CaBP as assessed by IHC (and in comparison to the HEK-CB12 cell lines) was very low. Therefore, an alternate expression vector with a

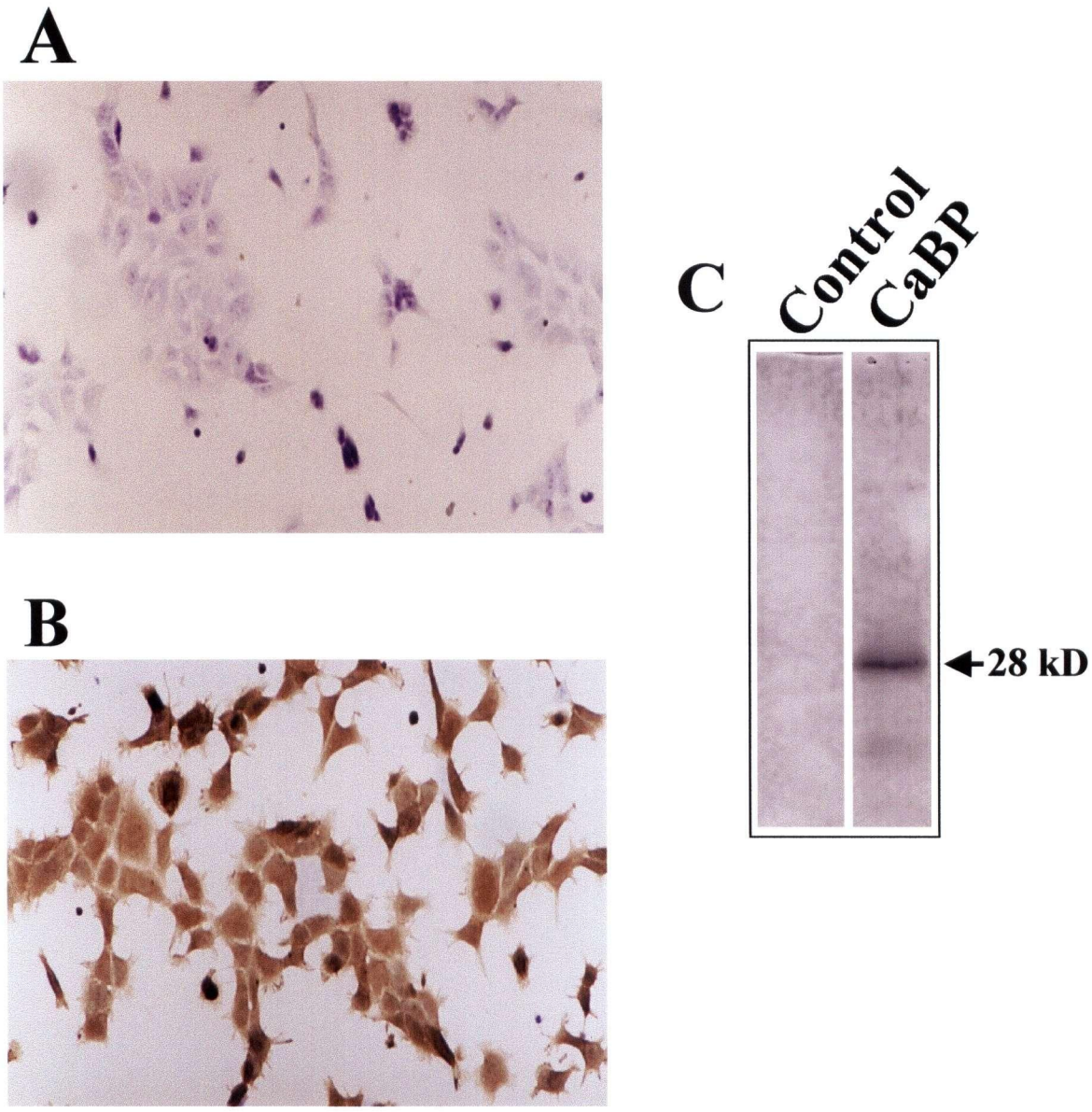


Figure 5. Expression of CaBP in an HEK cell line. A) Control cell (HEK-p3) line stably transfected with the pCDNA3 vector. Cells were stained for CaBP and counter-stained with cresyl violet. No expression of CaBP was evident in control or untransfected cell lines. B) Photomicrograph of HEK-CB12 cell line. Cells were fixed and stained for CaBP by IHC. C) Western blot of soluble protein extracts from HEK-CB12 and HEK-p3 cell lines.

modified CMV promoter was employed. The CaBP gene was inserted into the Xho1 and Xba1 sites of the mammalian expression vector pCINeo (Promega). Generation of clonal lines of HeLa cells containing the pCINeo-CaBP construct or the empty pCINeo vector (HeLa- pCINeo) was carried out as described above. Expression of CaBP from the pCINeo vector in HeLa cells was better than that observed for the pCDNA3 vector. As assessed by the intensity of IHC staining, the highest expressing clone (HeLa-CaBP), was selected for subsequent studies.

Estimation of CaBP Concentration in Transfected Cells

The concentration of CaBP in transfected cells was estimated using a western slot-blot method (Hersham et al., 1993). Transfected cells, grown on 10-cm tissue culture plates, were dislodged by trypsinization in 10 mM phosphate-buffered saline (pH 7.4). Live cells were counted using trypan blue exclusion, and replicate volumes containing at least 10^6 cells were centrifuged at 600 g for 3 min at room temperature. Cell pellets were stored at -20°C. For slot-blot analysis, cell pellets were resuspended in 500 μL of 50 mM Tris-buffered saline (TBS) containing 1 mM EDTA, boiled for 5 min, and centrifuged in a microfuge for 10 min. The supernatant was collected and the concentration of total soluble protein (TSP) was determined by A_{280} and A_{260} measurements. Correction for nucleic acid contamination was accomplished through use of the equation (Peterson, 1983):

$$[\text{Protein}]\ (\text{mg/mL}) = (A_{280} \times 1.55) - (A_{260} \times 0.76)$$

Aliquots (200 μL) containing from 0.05-20 μg of TSP were applied to a nitocellulose membrane using a Bio-Rad slot-blot apparatus according to the manufacturer's instructions. For standards, we used the recombinant human CaBP (see above) in the range of 0-1000 ng in addition to TSP extracted from a rat cerebellar homogenate.

After nitrocellulose membranes had been blocked overnight at 4°C in TBS containing 2% skim milk, 50% horse serum, and 0.2% Tween-20, they were incubated with polyclonal antibodies against CaBP (raised in rabbits against purified bovine cerebellar CaBP; Buchan and Baimbridge, 1988) at a dilution of 1:1,000 in TBS-Tween for 1 h at room temperature. Blots were rinsed twice in TBS-Tween (5 min each), and the secondary antibody (donkey anti-rabbit IgG conjugated with horseradish peroxidase; Amersham, Arlington Heights, IL, U.S.A.) was applied at a dilution of 1:2,000 for 1 h at room temperature. After two additional washes, Amersham's enhanced chemiluminescence system was used to visualise the CaBP protein. The amount of CaBP in cell extracts was determined according to the method of Hersham et al. (1993). Autoradiographs were scanned on an Eagleeye imaging system (Stratagene) and densitometry was carried out with Stratagene EagleSight software (Fig. 6).

The concentration of protein for HEK-CB12 and HeLa-CaBP cell lines were estimated to be 18 and 3.5 μg/mg TSP respectively. The rat cerebellar standard yielded a concentration of 24 μg/mg TSP. In the rat cerebellum, only Purkinje cells and climbing fibers contain CaBP, therefore this value is likely an underestimation (in proportion to the relative volume of Purkinje cells and climbing fibers to total cerebellar volume). The average expression level of CaBP in the transfected cell lines is therefore less than that

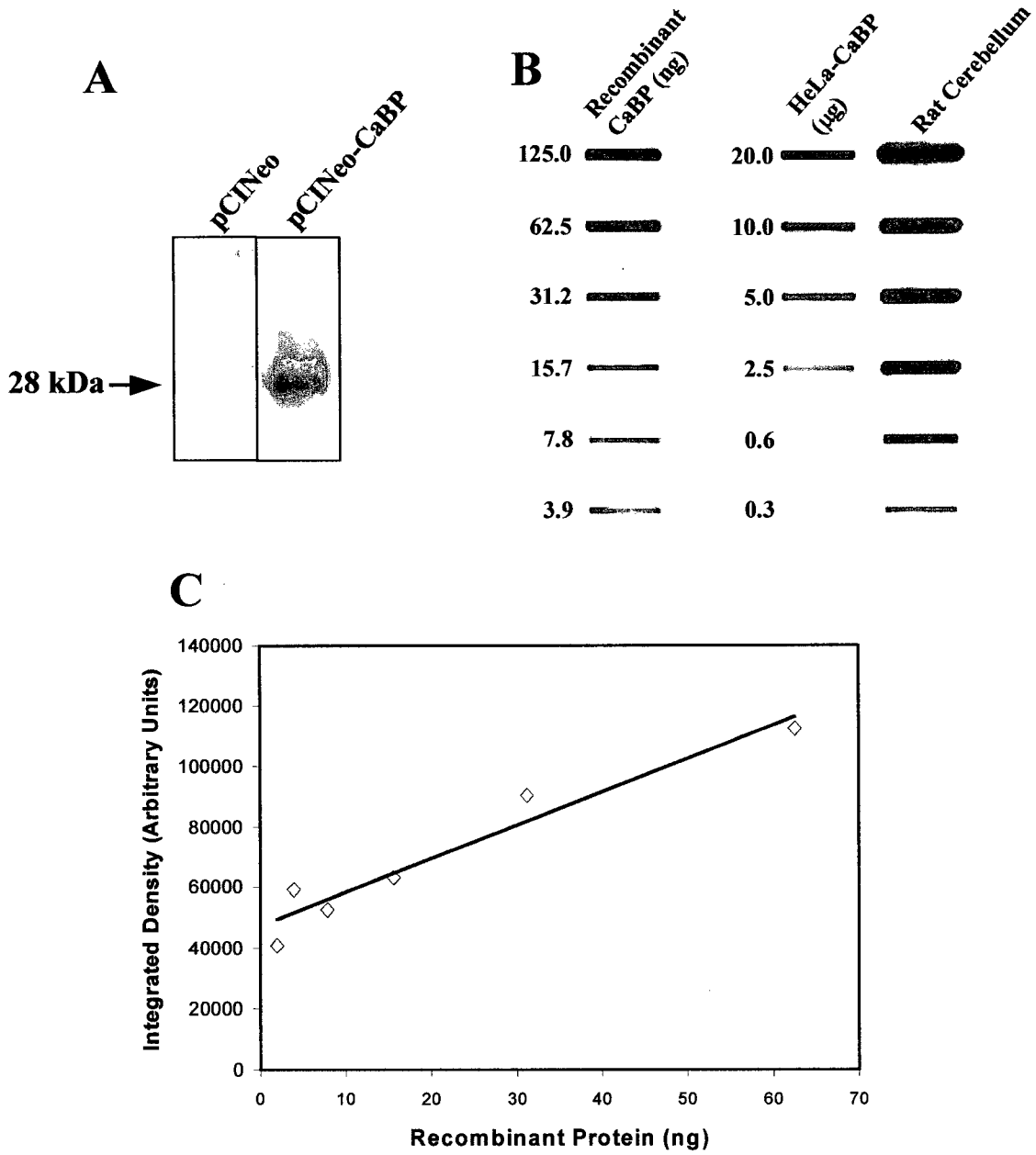


Figure 6. Quantification of CaBP Expression in Stably Transfected Cells. A) Western blot analysis of soluble protein extracted from HeLa-CaBP and HeLa-pCINeo cell cultures. B) Quantitative ECL slot blot analysis of HeLa-CaBP cells cultures. Recombinant CaBP standards were blotted with TSP extracts from HeLa-CaBP cells and (for comparison) with equivalent quantities of rat cerebellum TSP. C) Standard curve generated from densitometry of recombinant CaBP standards.

expressed naturally in Purkinje cells, but probably equivalent to or higher than levels expressed in other neurons (Baimbridge et al., 1992).

Transient Expression of the NMDA Receptor

Two separate constructs were employed to co-express the NR1 and NR2A subunits of the NMDA receptor (Raymond et al., 1996), both under control of the strong CMV promoter. Eight hours prior to transfection, cells were plated at a density of $1x10^6$ cells /10 cm plate. Transient transfection with 3μg of each NMDA subunit was carried out using Lipofectamine (Life Technologies) according the manufacturer's instructions. Following a 5 hour transfection period cells were washed twice with medium and replated on poly-D-lysine coated 22 mm circular glass coverslips. Cultures were incubated for a further 24h in the presence of 1 mM DL-2-Amino-5-phosphonopentanoic acid (APV) (Precision Neurochemicals; North Vancouver, BC) to protect transfected cells from excitotoxic cell death (Raymond et al., 1996), prior to loading with fura-2 and imaging.

Measurement of Intracellular Calcium : NR1/NR2A Transfected Cells

Two intracellular Ca^{2+} indicators were employed in these experiments; fura-2 and fluo-3. Fura-2 has the advantage of being a ratiometric dye and therefore facilitates estimation of intracellular $[Ca^{2+}]_i$. However, the quantum yield of fura-2 is much lower than that of fluo-3 (a non-ratiometric dye) and therefore necessitated higher loading concentrations. Therefore in experiments which were sensitive to the presence of high levels of Ca^{2+}-indicators (discussed in Chapter 4), fluo-3 was used instead of fura-2.

Transiently transfected cells were loaded with fura-2 by incubation at room temperature for 30 minutes in a balanced salt solution (BSS; NaCl, 139 mM; KCl, 3.5 mM; Na_2HPO_4, 3 mM; $NaHCO_3$, 2 mM; HEPES acid, 6.7 mM; HEPES-Na, 3.3 mM; D-glucose, 11 mM; $CaCl_2$, 1.8 mM; $MgCl_2$, 1 mM; Na pyruvate, 5 mM; glycine 2 μM; pH 7.35) containing 6 μM fura-2-AM (Molecular Probes), 0.05% bovine serum albumin and 1 mM APV. Fura-2 imaging was performed using a Zeiss-Attofluor™ digital fluorescence imaging system, as previously described (Abdel-Hamid and Baimbridge,1997). Coverslips of transfected cells were mounted in a heated chamber and superfused with BSS containing 100μM APV at 37°C for 15 min prior to any data collection. The ratio of fluorescence of fura-2 at 334/380 nm excitation (corrected for background fluorescence) was determined at a rate of 1 s^{-1} using a 63x objective in individual HEK cells, before, during, and for 10 minutes following superfusion of the cells with BSS containing 500 μM glutamate. Parameters of the glutamate-induced Ca^{2+} transient examined were the rate of rise, peak response and rate of recovery. Fura-2 measurements are expressed in terms of fluorescence ratios. When calibrated by the

methods of Grynkiewicz et al. (1985), ratios of 0.5, 1.0, 2.0 and 3.0 were calculated to correspond to $[Ca^{2+}]_i$ values of 80.8 nM, 280.9 nM, 783.8 nM and 1.4 μM respectively.

Measurement of Intracellular Calcium : Flash Photolysis

In another series of experiments, HEK-CB12 and HEK-p3 cells were loaded with the fluorescent indicator fluo-3 or fura-2 and the caged calcium compound NP-EGTA (Ellis-Davies and Kaplan, 1994) by the use of their respective cell permeant acetoxy-methyl ester derivatives (Molecular Probes) as previously described (Sidky and Baimbridge, 1997). Briefly, individual coverslips plated with HEK-CB12 or HEK-p3 cultures were incubated for 1 hour at room temperature in the dark in BSS containing 3.1 μM fluo-3-AM or 6 μM fura-2-AM and 8 μM NP-EGTA-AM. They were then transferred to fresh BSS and incubated at room temperature for at least 10 minutes prior to mounting on the imaging chamber. Flash photolysis of NP-EGTA, achieved by a 0.4 s exposure to unfiltered UV light, results in a rapid and transient increase in $[Ca^{2+}]_i$ which is relatively uniform throughout the cytoplasmic volume (Ellis-Davies and Kaplan, 1994). Cells which did not display at least a two-fold increase in background-corrected fluorescence intensity (fluo-3) in response to flash photolysis were excluded from further analysis. Fluo-3 fluorescence intensity (in the range of 0-255 arbitrary units) was determined after flash photolysis in individual cells (up to 99 in a single field of view) for up to 10 minutes at a rate of 1 s^{-1} for the first 3 minutes and thereafter at a rate of 0.5 s^{-1}. For each cell, the background-corrected fluorescence intensity was normalized

between a value of 0 and 1 with 0 being the baseline value to which fluo-3 fluorescence decayed, and 1 being the fluorescence at the first time-point immediately (~ 0.6 s) following photolysis. Data collection was carried out in a similar fashion for fura-2 loaded cells.

Imaging Calcium Oscillations in HeLa Cells

To capture Ca^{2+} oscillations, HeLa cells were loaded with fura-2 or fluo-3 as described above, and imaged at a rate of 1 s^{-1} using a 63x objective. Individual cells (up to 50 in a single field of view) were monitored before during and after a 300 s superfusion with BSS containing 1 μM histamine for 300 s. These experiments were all performed at room temperature.

Calcium Wave Propagation Analysis in HeLa Cells

To investigate calcium wave propagation velocity, individual elongated HeLa cells were selected for analysis and imaged using a 100x objective. A single pixel wide line was selected along the length of the HeLa cell and the fluorescence along that line was measured at a rate of 15 images s^{-1} for approximately 30s. Fluorescence intensity values were corrected for background and converted to %ΔF/F. A criterion of an

increase in fluorescence of 50% of the maximum value was selected as the threshold for the determination of the time course of a calcium wave along the scanned region. Wave velocity was then determined by plotting the time point at which specific pixels along the scanned line reached the 50% $\Delta F/F$ value.

Image Analysis & Statistical Methods

All imaging data analysis was carried out in Microsoft Excel employing custom designed macros written in Visual Basic™ by this author. Statistical comparisons between treatment groups were performed using unpaired t-tests.

Excitotoxicity Studies

HEK stable cell lines were transfected with NR1/NR2A and the marker plasmid pCMVβ (Clontech) as described above. Following removal of the transfection mixture, each 10cm plate of transfected cells (either HEK-CB12 or HEK-p3) were split evenly among the wells of a 6-well tissue culture plate. Cells were allowed to attach to the plates for 18 hrs in DMEM containing 1mM APV prior to excitotoxic treatments. Cultures were washed twice with a bicarbonate-buffered salt solution, (BBSS; 140 mM NaCl, 1.4 mM $CaCl_2$, 5.4 mM KCl, 1.2 mM NaH_2PO_4, 21 mM Glucose, 26 mM

$NaHCO_2$; pH 7.4), then incubated in BBSS containing 200 μM NMDA at 37° in an atmosphere of 5% CO_2 for 1 hour. In control cultures (3 wells of the six well plate) 1mM APV was substituted for NMDA, but they were otherwise treated identically. Following treatment, cells were washed twice with BBSS then incubated for a further 6 or 24 hrs in fresh DMEM with 1mM APV (37° 5% CO_2). Cells were then fixed (4% formaldehyde, 0.2% glutaraldehyde in PBS; 5 min room temperature) and stained for β-galactosidase activity. Surviving cells which had been successfully transfected (as assessed by the presence of β-galactosidase staining) were counted by examining 21 fields (20x objective) across each well of the tissue culture plates.

In excitotoxicity experiments, to assess cell death, remaining cells were counted. This relies upon the assumption that dead cells will detach from plates. This assumption was made based upon initial observations of plates co-transfected with NR1, NR2A and the marker plasmid pCMVβ, in which no NMDA receptor inhibitor was added. In these experiments, no cells positively stained with the β-gal marker plasmid remained attached to the plate.

Chapter 3

Effect of Recombinant CaBP on Intracellular Ca^{2+} Transients in Stably-transfected HEK 293 Cells

Chapter Summary

1. The effect of stable CaBP expression in HEK 293 cultures on changes in $[Ca^{2+}]_i$ induced by a variety of stimuli was investigated.

2. HEK 293 cells expressing the NR1/NR2A subunits of the NMDA receptor responded to brief glutamate stimulation with Ca^{2+} transients that were prolonged in cells expressing CaBP.

3. Ca^{2+} transients induced by treatment with the ionophore 4-Br-A23187 displayed an attenuated rate of rise and peak $[Ca^{2+}]_i$ and a prolonged recovery in cells expressing CaBP.

4. Flash Photolysis of NP-EGTA resulted in a rapid release of intracellular Ca^{2+} which required longer periods of time to dissipate in cells expressing CaBP.

5. Exposure of HEK 293 stable cell lines to ATP resulted in Ca^{2+} transients in which the rate of rise and peak $[Ca^{2+}]_i$ was reduced in cells expressing CaBP.

6. Capacitative Ca^{2+} influx was induced by treatment with thapsigargin and modulation of external Ca^{2+}. The resulting rate of rise in $[Ca^{2+}]_i$ was reduced in cells expressing CaBP.

7. In studies of delayed, Ca^{2+}-dependent cell death, CaBP transfected cells exhibited enhanced survival 24 hours after a 1 hour exposure to 200 μM NMDA. However, acute, necrotic cell death observed after the first 6 hours was not prevented by the presence of CaBP.

8. These results therefore provide direct evidence for a Ca^{2+}-buffering effect of CaBP which serves to protect cells from death mediated most likely by apoptosis.

Introduction

It has been hypothesized that CaBP acts as an intracellular Ca^{2+} buffer, and that one of the consequences of this action is to enhance cell survival in Ca^{2+} mediated cytotoxicity. To address this hypothesis, a number of studies have examined the effect of CaBP on Ca^{2+} transients utilizing whole cell Ca^{2+} imaging or electrophysiological techniques. Reductions in peak $[Ca^{2+}]_i$ and rate of decay of Ca^{2+} transients induced by depolarization have been reported in neurons in which CaBP was injected by patch pipette (Chard et al., 1993) and in retrovirally transfected GH_3 cells (Lledo et al, 1992). In CaBP null mutant mice studies, peak $[Ca^{2+}]_i$ was lower and recovery of synaptically evoked postsynaptic Ca^{2+} transients were *slower* in wild-type (CaBP+) mice (Airaksinen et al, 1997). Peak $[Ca^{2+}]_i$ response reduction has also been reported in glutamate-stimulated neurons transfected with CaBP using a viral vector (Meier et al, 1998) and in CaBP-transfected PC12 cells exposed to ATP (McMahon, 1998). Consistent with many of the studies cited above, the decay from the peak $[Ca^{2+}]_i$ was also prolonged in neurons naturally expressing CaBP (Abdel-Hamid et al, In Preparation), however these neurons displayed an increased peak $[Ca^{2+}]_i$ in response to NMDA.

The experiments described in this chapter systematically examined the effects of CaBP on Ca^{2+} transients. The objective was to examine the effect of stably-transfected CaBP upon intracellular Ca^{2+} transients originating from different sources (intracellular vs extracellular) and having different temporal and spatial characteristics (Ca^{2+} gradient vs global release).

Initial experiments were an extension of our recent electrophysiological investigation of the effect of CaBP upon transfected NMDA receptor activity (Price et al., 1999). It was found that in cells transfected with CaBP there was a significant slowing of the development of Ca^{2+}-dependent rundown of peak glutamate-evoked current. This effect was presumed to be due to the buffering action of CaBP, although a protein-protein interaction cannot be ruled out as a possible mechanism for modulation of Ca^{2+} influx.

To generate an influx of Ca^{2+} with similar dynamics but utilizing a structurally unrelated molecule, stable cells were exposed to the ionophore 4-Br-A23187. To assess Ca^{2+} buffering on Ca^{2+}-transients with different dynamics, flash photolysis of the caged Ca^{2+} compound, NP-EGTA, was employed. Use of NP-EGTA allowed us to examine the effect of a global, nearly instantaneous release of Ca^{2+}, independent of internal or external stores. To examine the possible effects of CaBP upon release of internal stores of Ca^{2+}, ATP was used to activate the PLC-IP_3 pathway, and subsequently release Ca^{2+} from the ER. This likely produces gradients of Ca^{2+} across the cell due to sequential activation of IP_3 receptors. Finally, many recent studies have focused on Ca^{2+} influx stimulated by emptying of internal stores or "capacitative" Ca^{2+} influx (see reviews by Putney, 1999; Berridge, 1995). The mechanism through which this operates has yet to be elucidated, but likely involves a plasma membrane channel. By emptying intracellular stores with thapsigargin, we examined the effect of CaBP on capacitative Ca^{2+} entry.

Results

Characterization of Ca^{2+} Transients in NR1/NR2A Transfected HEK 293 Cultures

To examine the effect of transfected CaBP upon NMDA receptor mediated changes in $[Ca^{2+}]_i$, HEK-CB12 and HEK-p3 cells were transiently transfected with the NR1 and NR2A receptor subunits. While measuring changes in $[Ca^{2+}]_i$ with fura-2 or fluo-3, cells were exposed for 20s to 500μM glutamate, followed by rapid washout.

Following transfection with the NR1/NR2A subunits, cells which successfully expressed functional NMDA receptors were identified by ratiometric imaging on the basis of their response to 500μM glutamate. It was observed that variable magnitudes of Ca^{2+} transients were generated in response to glutamate, likely due to differing levels of NMDA receptor subunit expression (Raymond et al., 1996). As described in previous studies of NMDA receptor transfected HEK 293 cells, the continuous presence of an NMDA channel antagonist in the culture medium was necessary following transfection to prevent cytotoxic cell death by inappropriate channel activation (Raymond et al., 1996). This is likely due to the fact that in neurons, NMDA receptor operated channels demonstrate a voltage-dependent block by Mg^{2+} , whereas the resting membrane potential of HEK cells are in the range where Mg^{2+} blockade is relieved (ie < -50mV). In accord with this observation, during Ca^{2+}-imaging experiments, a slow increase in $[Ca^{2+}]_i$ was observed when no antagonist was present (data not shown). Therefore during all imaging experiments, 100 μM of the competitive antagonist APV was present throughout. Under these conditions, short exposure (<20s) to glutamate resulted in a

rapid increase in $[Ca^{2+}]_i$ to a peak value followed by a slow recovery upon washout. It was observed that maximal increase in $[Ca^{2+}]_i$ was achieved with doses of glutamate between 500 μM and 1mM (Fig. 7).

Effect of CaBP on Glutamate Treated HEK Cells Transfected with NR1/NR2A

From transfection to transfection and from cell to cell, variable magnitudes of Ca^{2+} transients were produced in response to glutamate, presumably due to differing levels of NMDA receptor subunit expression. Similar variations in CaBP expression levels were noted in *transiently* transfected CaBP in HEK cells. For the following experiments therefore, we utilized stable cell lines to alleviate this variability.

Fig. 8 depicts two representative results from a typical Ca^{2+} imaging experiment involving a 20s, 500 μM glutamate treatment of HEK-p3 and HEK-CB12 cells which have been transiently transfected with the NR1/NR2A subunit combination. Responses from a total of 107 control HEK-p3 cells and 104 HEK-CB12 cells were analyzed. No significant difference was observed in the rate of 334/380 ratio increase (HEK-p3: 0.117±0.01; HEK-CB12: 0.128±0.012 ratio units s^{-1}). The peak ratios of the HEK-p3 (2.46±0.13) and HEK-CB12 (2.41±0.09) cell lines were not significantly different. However, in the recovery to baseline levels the two cell types differed significantly. When the Ca^{2+} response of each cell was normalized to the peak and baseline value, the recovery times to 75%, 50% and 25% of the maximum were found to be significantly longer in the CaBP expressing cells (HEK-CB12: 75%=31.4±3.2 s, 50%=111±10.8 s,

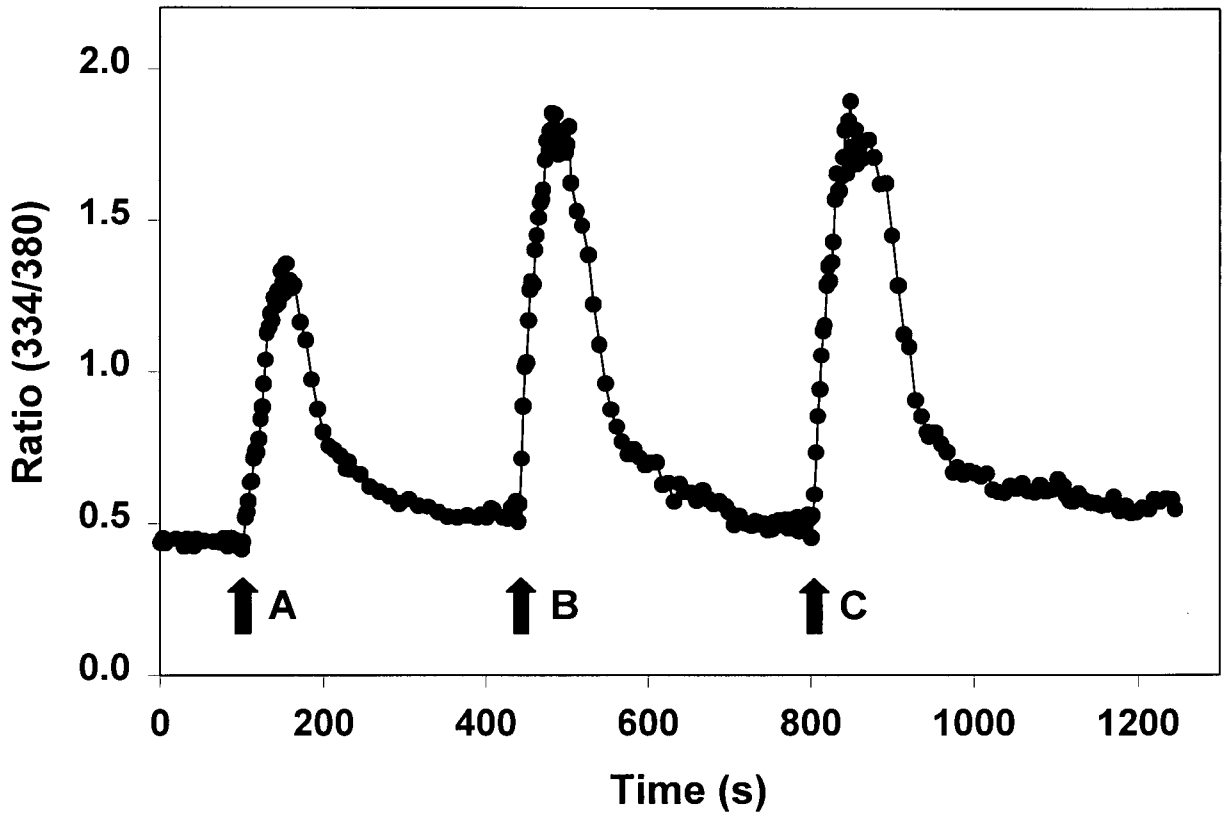


Figure 7 **Response of NR1/NR2A transfected HEK 293 cells to glutamate.** Mean $[Ca^{2+}]_i$ response of 4 HEK 293 cells transiently transfected with NR1/NR2A subunits of the NMDA receptor and briefly exposed to (A) 200μM (B) 500μM and (C) 1mM glutamate. $[Ca^{2+}]_i$ is expressed as the ratio of fura-2 fluorescence at 334 and 380 nm excitation.

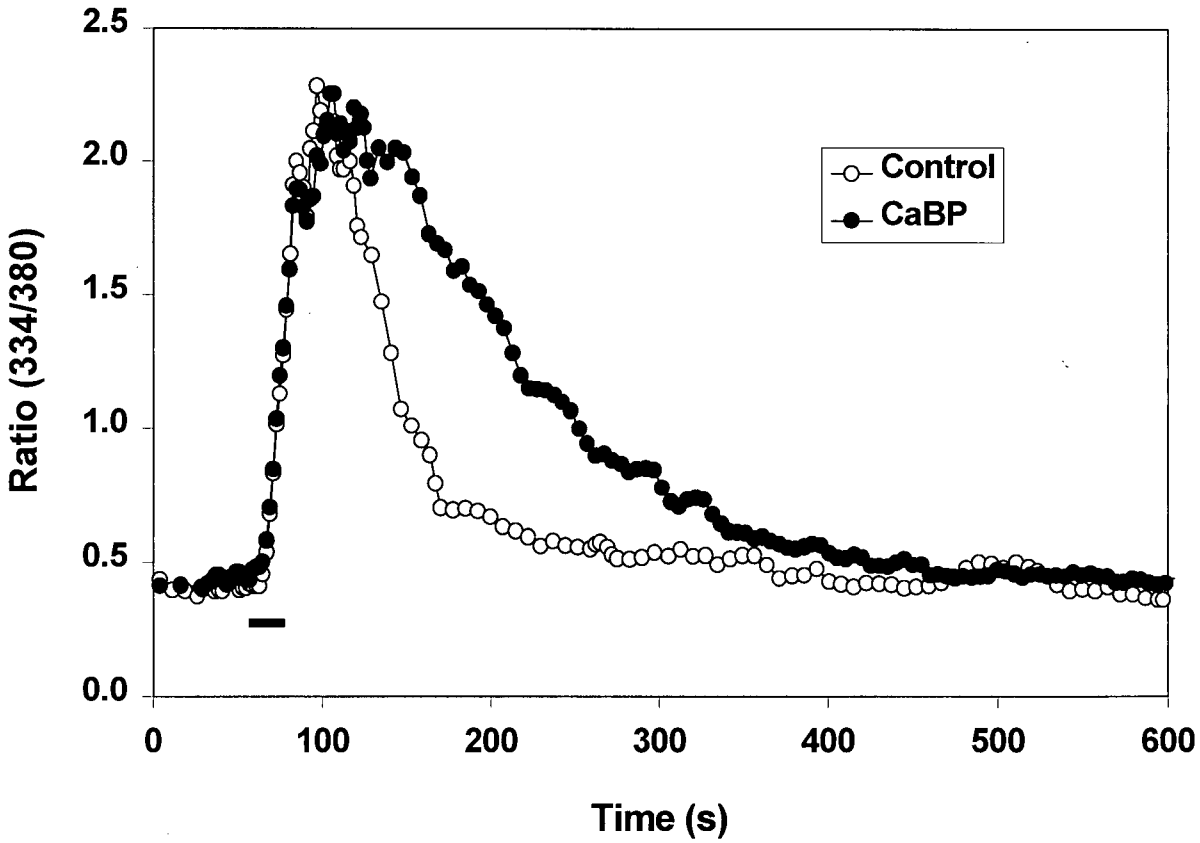


Figure 8 **Response of HEK-CB12 and HEK-p3 cells transiently transfected with NR1/NR2A to treatment with glutamate**. Ratio of fura-2 fluorescence in HEK 293 cells transiently transfected with NR1/NR2A. Shown is a representative response of an HEK-p3 cell compared with that of an HEK-CB12 cell. Cells were superfused for 20s with 500 μM glutamate at 37°

25%=220.3±14.9 s N=104; HEK-p3: 75%=17.7±1.5 s, 50%= 57±7.9 s, 25%=134.1±11.7 s N=107) (Fig. 9).

Modulation of Br-A23187 Mediated Ca^{2+} Transients by CaBP

To eliminate the high variability of Ca^{2+}-responses due to the levels of NMDA receptor expression, the ionophore 4-Br-A23187 was applied to the stable HEK cell lines. 4-Bromo-A23187 is a non-fluorescent Ca^{2+} ionophore which has been used extensively to increase intracellular Ca^{2+} levels. Upon perifusion of fura-2 loaded HEK-293 stable cell lines with 2.5 μM 4-Br-A23187 for 100s, $[Ca^{2+}]_i$ increased rapidly to a peak which recovered towards baseline values following washout (Fig 10). No effect upon $[Ca^{2+}]_i$ was observed in control (0.75% DMSO) treated cells. When compared to HEK-p3 cells (N=53), HEK-CB12 cells (N=41) displayed a significant attenuation in the rate of rise (slope calculated between 25% and 75% of maximum ratio; HEK-p3 = 0.085±0.005 ratio units s^{-1}, HEK-CB12 =0.058±0.003 ratio units s^{-1}; $p<0.01$), peak ratio (Fig. 11A) (HEK-p3 $Ratio_{max}$ = 4.2±0.25, HEK-CB12 $Ratio_{max}$ =3.1±0.16; $p<0.001$), and decay (Fig. 11B) (HEK-p3 = -0.0117±0.0008 ratio units s^{-1}, HEK-CB12 =-0.0095 ±0.0005 ratio units s^{-1})

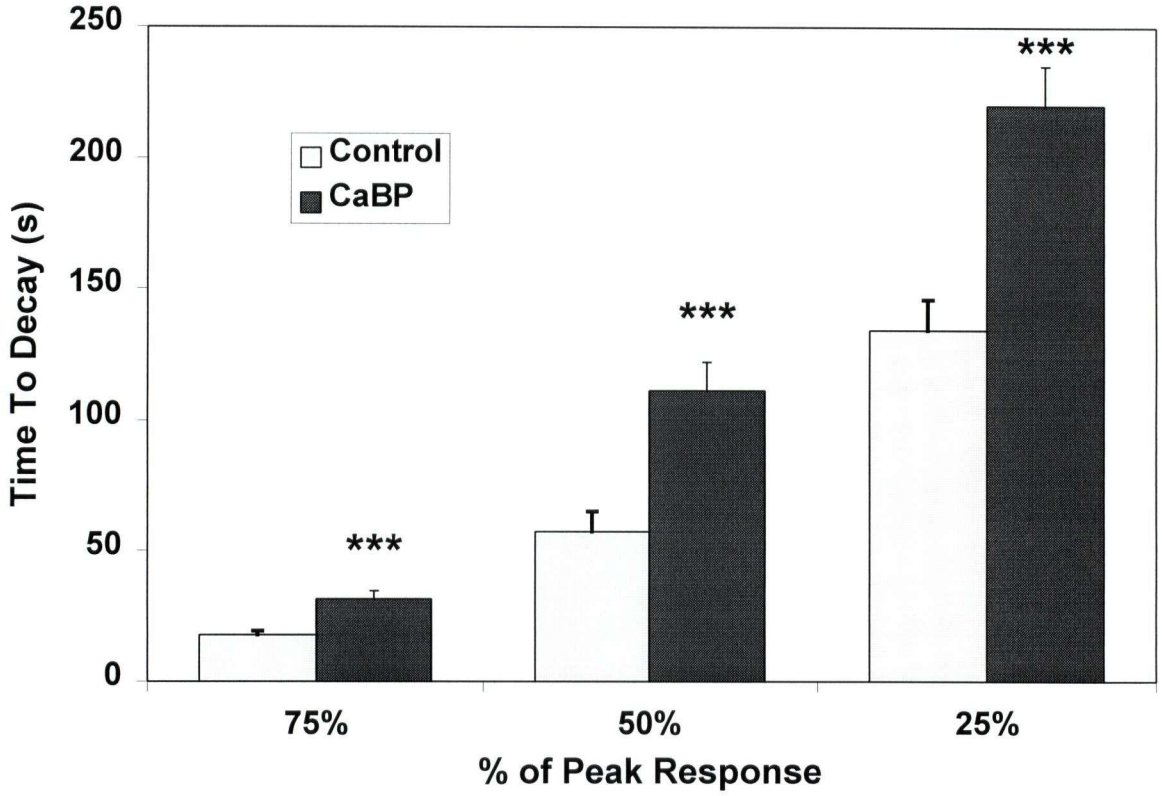


Figure 9 **Pooled data of recovery of stable HEK 293 cells transiently transfected with NR1/NR2A and stimulated with glutamate**. Bars indicate mean time to recovery to the percentage of maximal response indicated. Error bars indicate SEM. *** $p<0.001$. HEK-p3, N=107; HEK-CB12, N=104.

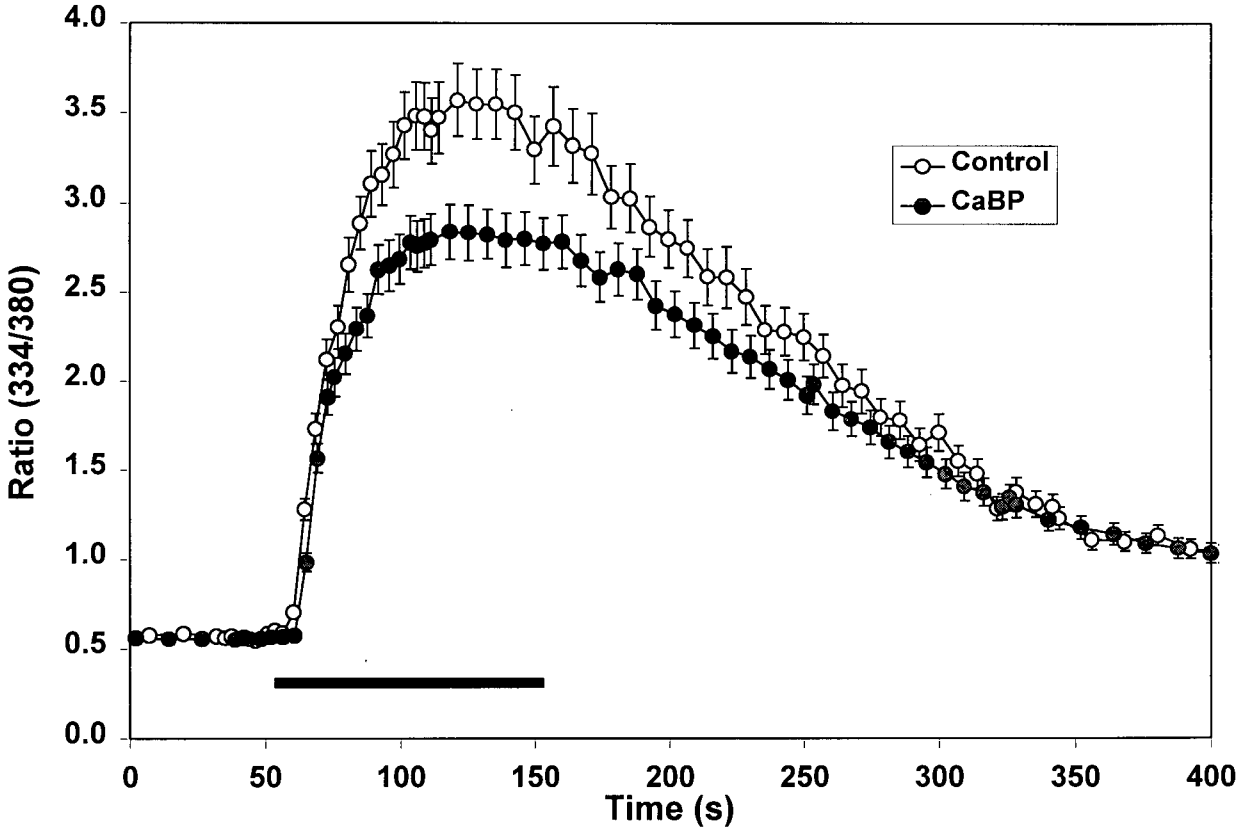


Figure 10 **Effect of CaBP upon 4-Br-A23187-induced Ca^{2+} transients in HEK293 cells.** Mean $[Ca^{2+}]_i$ responses of HEK cells from a single coverslip of either HEK-p3 (N=18) or HEK-CB12 (N=22) cells. Cells were exposed to a 100 s superfusion of 2.5 μM 4-Br-A23187 in BSS at 37°.

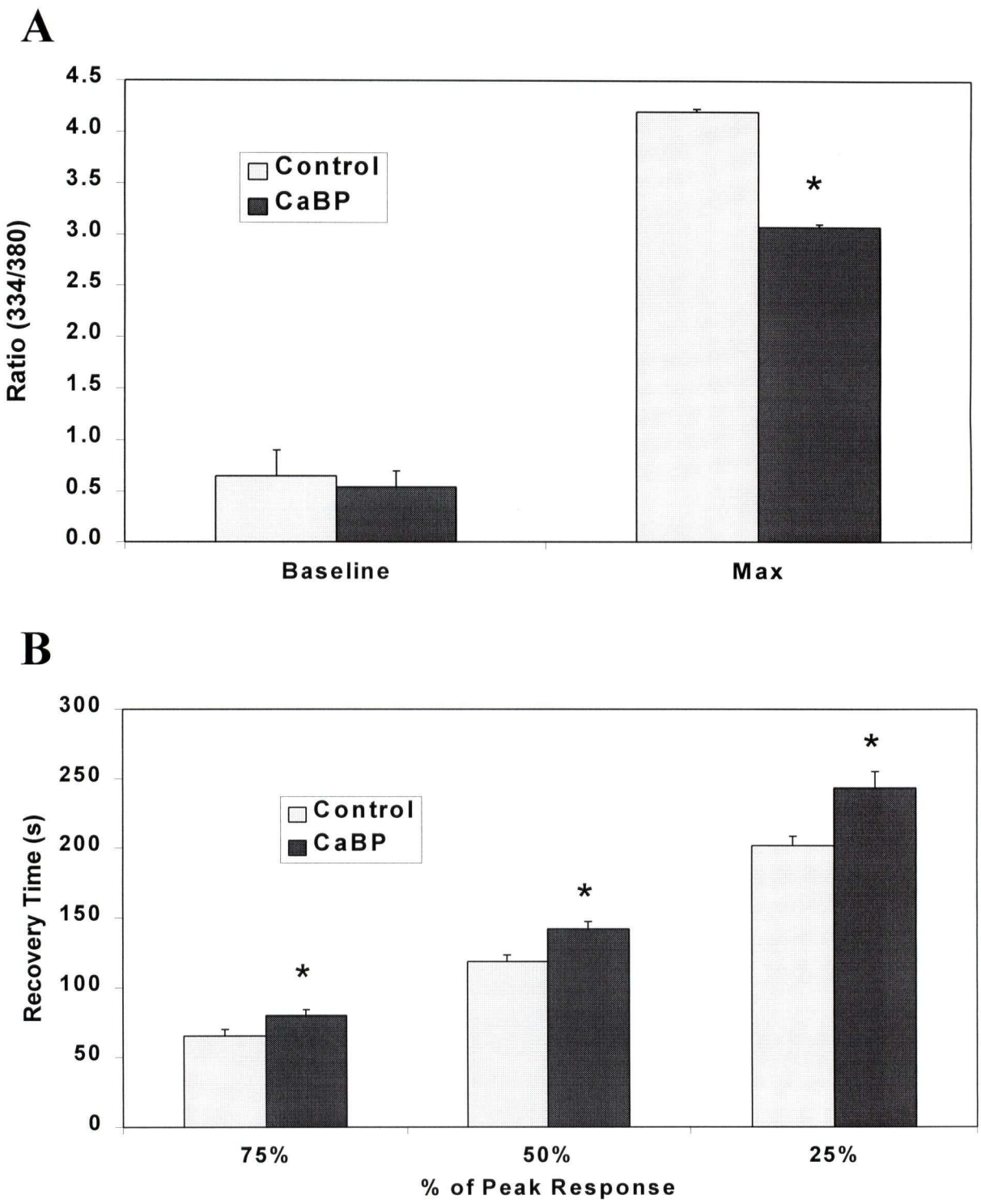


Figure 11 **Pooled calcium imaging data from stable HEK 293 cells treated with 4-Br-A3187.** A) Basal levels prior to, and mean maximum ratios following treatment with 4-Br-A23187. B) A total of 18 HEK-p3 cells were compared to 22 HEK-CB12 cells. The Y-axis denotes mean time to recovery to the percentage of the maximum response indicated on the X-axis. Error bars indicate SEM. * $p<0.05$.

Effect of Stably Expressed CaBP in HEK Cells Following Flash Photolysis of NP-EGTA

Both of the above experiments involved influx of extracellular Ca^{2+}, resulting in a gradient of Ca^{2+} extending from the channels/pores. NP-EGTA was utilized as a method of globally and nearly instantaneously releasing Ca^{2+} intracellularly. Initial experiments with this caged Ca^{2+} compound were carried out with the non-ratiometric dye fluo-3 to avoid inappropriate photolysis by UV wavelengths of light necessary for fura-2 ratiometric imaging. Since non-photolysed NP-EGTA can act as a Ca^{2+} buffer, near-complete photolysis by a 0.4s flash of unfiltered UV light was confirmed in control experiments involving sequential UV flash exposures, in agreement with Sidky and Baimbridge (1997).

In all experiments with NP-EGTA and fluo-3 loaded stable HEK 293 cells, flash photolysis of NP-EGTA resulted in an increase in fluo-3 fluorescence that was already declining from the time of the first measurement taken (~0.6s). All cells demonstrated a rapid initial recovery phase followed by a slower recovery to baseline. In HEK-CB12 cells, the initial fast-recovery phase was unaffected, however a pronounced "plateau" phase was evident in the initial phase of the slow recovery. Full recovery to baseline $[Ca^{2+}]_i$ was observed in both HEK-p3 and HEK-CB12 cell lines after about 250s (Fig. 12).

An obvious drawback of the above method is the non-quantitative nature of fluo-3 imaging. Attempts were therefore made to utilize fura-2, while minimizing unwanted photolysis of the caged Ca^{2+} compound. In preliminary experiments, it was found that a

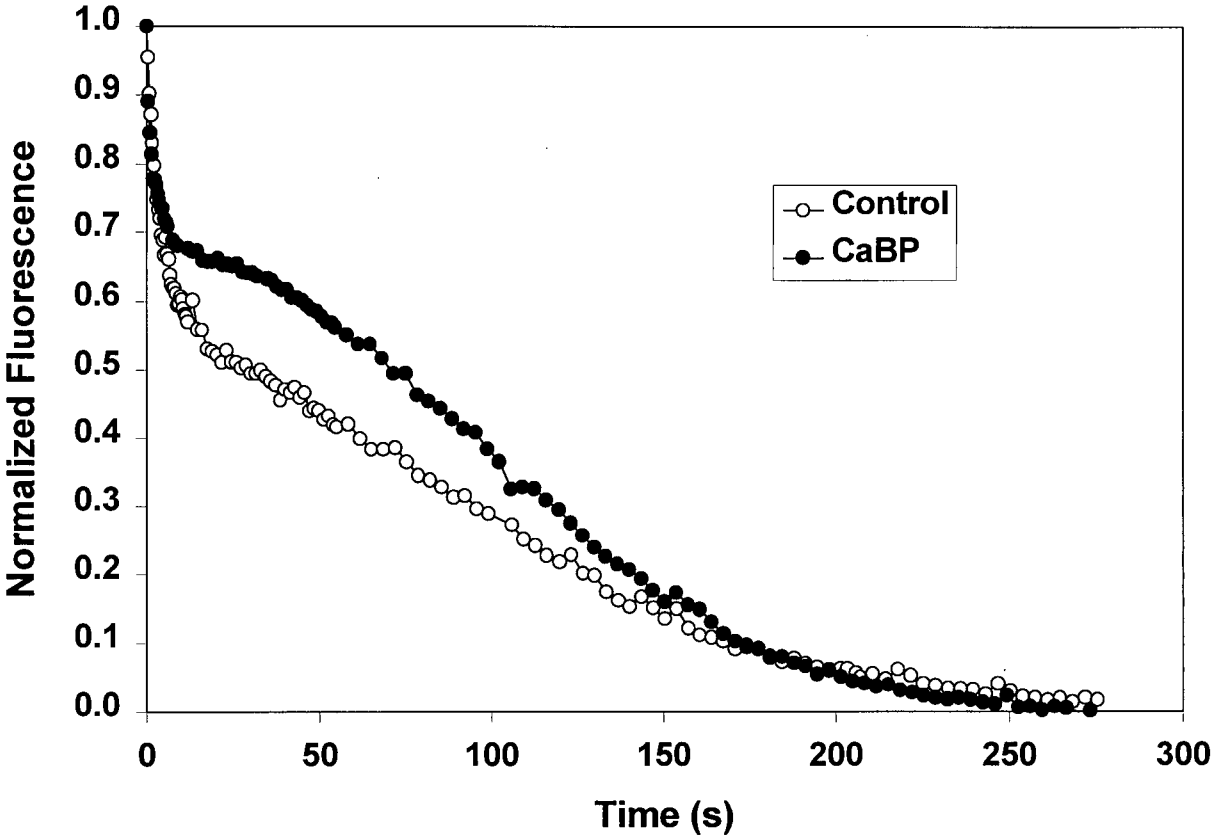


Figure 12 **Effect of CaBP on recovery following flash photolysis of NP-EGTA in fluo-3 loaded stable HEK cells.** Mean normalized intensity of fluo-3 fluorescence following flash photolysis of NP-EGTA in HEK-p3 cells (N=61) and HEK-CB12 cells (N=80).

single exposure of NP-EGTA loaded cells to the wavelengths of UV light necessary for fura-2 excitation (334 and 380 nm) resulted in minimal photolysis of NP-EGTA. Therefore, in fura-2 experiments, fields of cells loaded with NP-EGTA were limited to a single ratio collection (single exposure to 334 and 380nm light) prior to flash photolysis with unfiltered UV.

The pattern of the $[Ca^{2+}]_i$ response to NP-EGTA photolysis in fura-2 loaded cells (Fig. 13) was essentially the same as that seen with fluo-3. However, fura-2 loaded cells required longer to recover to baseline than fluo-3 loaded cells. This may be a result of additional buffering by fura-2, as cells were loaded with more fura-2 (6 μM loading concentration) than fluo-2 (3.1 μM loading concentration). In comparing HEK-CB12 and HEK-p3 cells loaded with fura-2, peak ratios achieved upon uncaging were not statistically different ($p>0.05$). Average peak ratios were 2.28±0.16, N=8 and 2.54±0.17, N=9 (334/380 ratio units) for HEK-p3 and HEK-CB12 cultures respectively. Using the calibration described in Chapter 2 these ratios would correspond to 1.04 μM and 1.07 μM $[Ca^{2+}]_i$ respectively. As with the fluo-3 loaded cells there was a significant prolongation of $[Ca^{2+}]_i$ in the recovery phase (Fig. 14), with the time to decay to 50% and 25% of the maximum ratio significantly longer in the HEK-CB12 cells (44.72±10.9 s and 191.1±18.4 s, N=8) as compared to the HEK-p3 cells (14.1±3.9 s and 134.3±16.5 s, N=9).

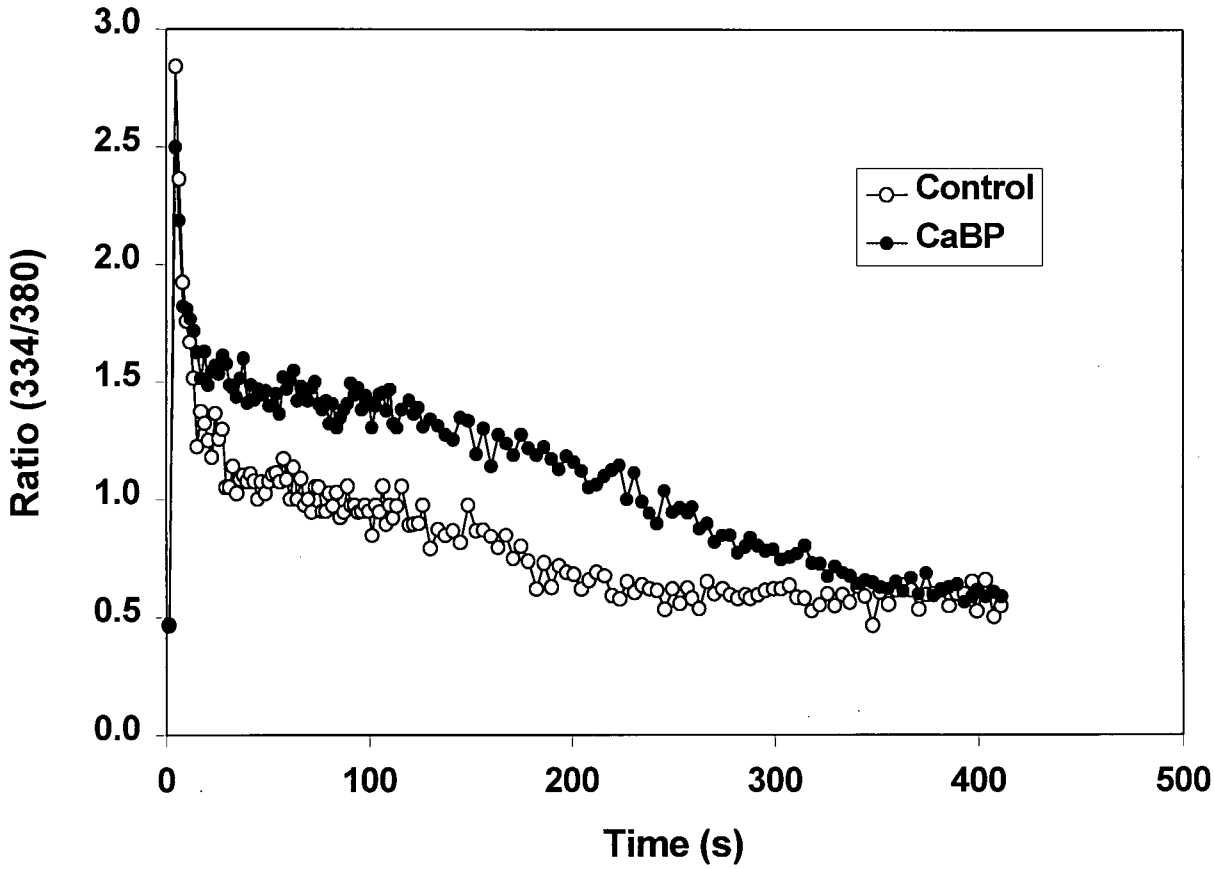


Figure 13 **Effect of CaBP on recovery following flash photolysis of NP-EGTA in fura-2 loaded stable HEK cells.** A single ratio acquisition was followed by a 0.4s exposure to unfiltered UV light. Shown are representative responses of single HEK-p3 and HEK-CB12 cells.

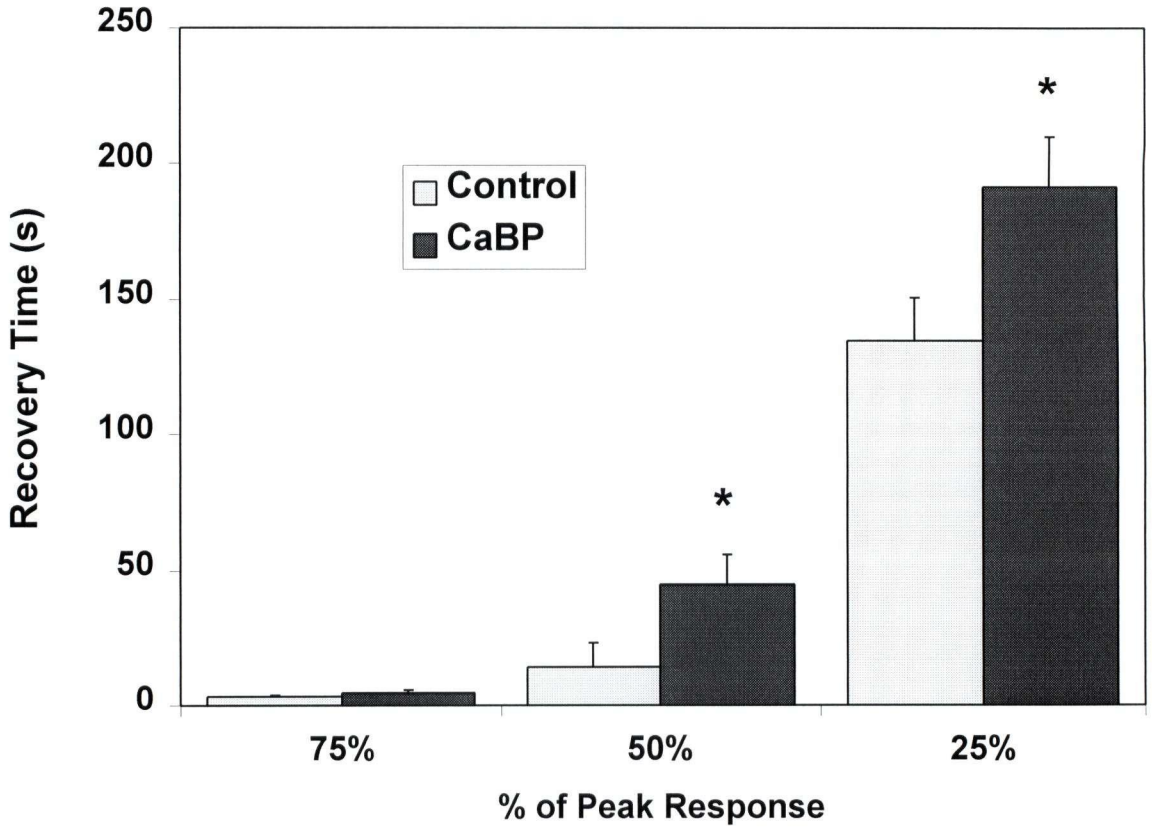


Figure 14 **Pooled data of recovery from NP-EGTA released Ca^{2+} in fura-2 loaded stable HEK cell lines.** Bars indicated mean time to recovery to the percentage of maximal response indicated. Error bars indicate SEM. * $p<0.05$.

Ca^{2+} Imaging of Stably transfected HEK cells treated with ATP

To determine the effect of CaBP on Ca^{2+} released from internal stores, $[Ca^{2+}]_i$ was monitored with fura-2 in stable HEK cells treated with ATP. ATP likely acts by binding to a P-type purinergic receptor which subsequently activates the PLC-$InsP_3$ pathway, inducing release of Ca^{2+} through IP_3 receptors in the ER (Bischof et al., 1997).

HEK-CB12 and HEK-p3 cells exposed to a continuous superfusion of 10μM ATP exhibited immediate increases in $[Ca^{2+}]_i$, to a peak followed by a rapid recovery to basal levels, despite the continued presence of the agonist. Representative results of such an experiment are shown in Fig. 15. Unlike $[Ca^{2+}]_i$ responses to NMDA receptor activation, ATP induced a marked $[Ca^{2+}]_i$ oscillatory response (Fig. 15A inset) which persisted until the removal of the agonist. In averaged Ca^{2+} responses of ATP treated cells (Fig. 15 B&C), significant reductions in the peak $[Ca^{2+}]_i$ value (HEK-p3 mean ratio: 2.88±0.09, N=44; HEK-CaBP mean ratio:2.51±0.07, N=49) and rate of rise (HEK-p3: 0.40±0.018 ratio units s^{-1}, N=44; HEK-CaBP 0.28±0.014 ratio units s^{-1}, N=49) were evident. In the initial recovery phase, HEK-p3 cells were significantly faster to recover, requiring less time (8.9±1.42 s N=44) than HEK-CB12 cells (24.5±3.16 s N=49) to recover to 75% of the maximum ratio. No significant differences were found in the later stages of recovery, however, the oscillatory patterns in $[Ca^{2+}]_i$, precluded a meaningful analysis of recovery times.

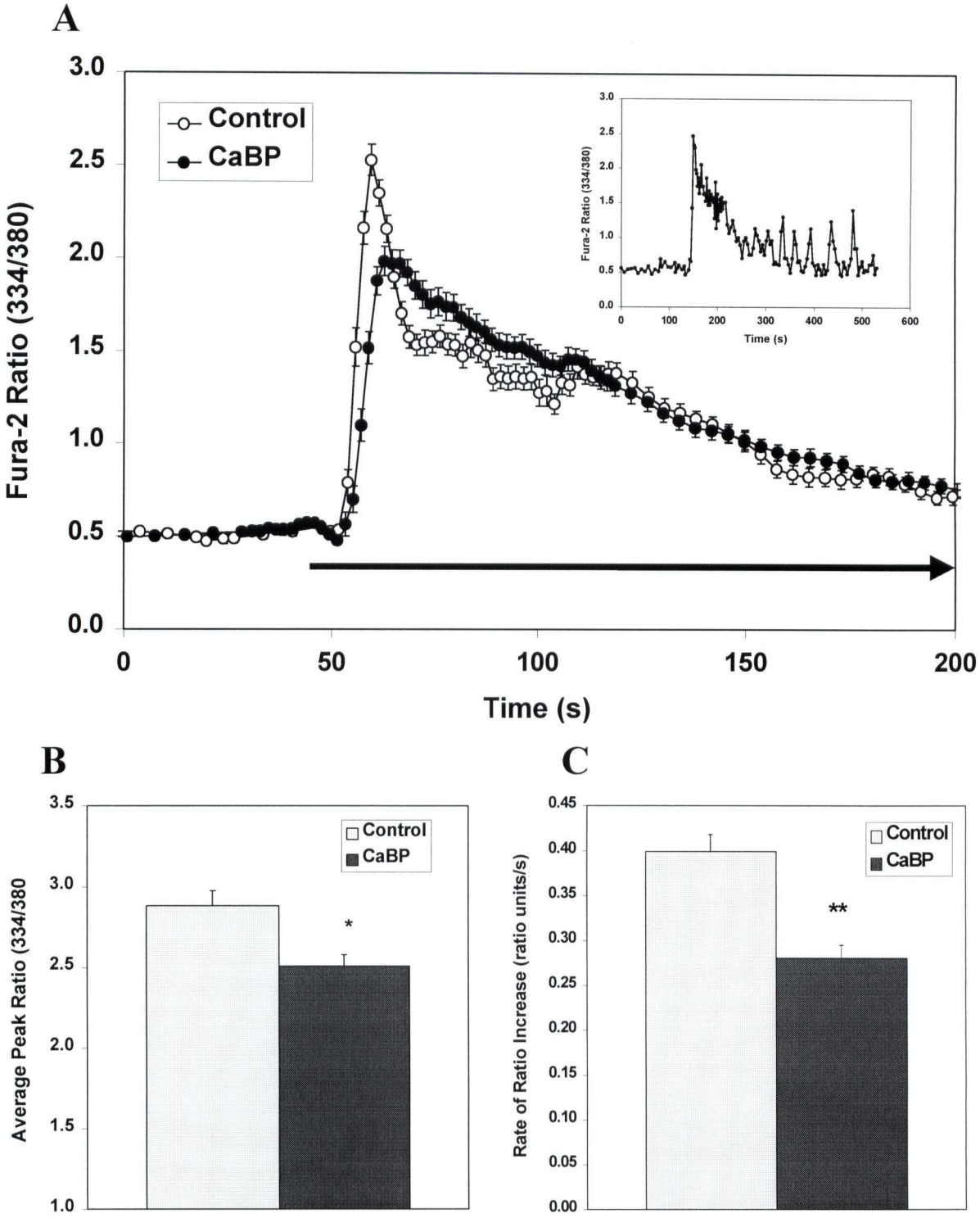


Figure 15 **Effect of CaBP upon ATP induced Ca^{2+} release in stable HEK 293 cells.** A) Average fura-2 fluorescence ratios of stably-transfected HEK 293 cell perfused with 10 μM ATP. Mean values of 44 HEK-p3 cells and 49 HEK-CB12 cells are shown. The horizontal bar indicates the 300s treatment period. Inset: Representative trace of a single HEK cell demonstrating Ca^{2+} oscillations.B) Mean maximum fura-2 ratios of stably-transfected HEK 293 cells in response to perfusion with 10μM ATP. C) Rate of increase in fura-3 fluorescence in stably transfected cells (HEK-p3 N=44, HEK-CB12 N=49) upon perfusion with 10 μM ATP. Error bars indicate SEM. * $p<0.05$. *** $p<0.001$

Effect of CaBP Upon Capacitative Ca^{2+} Influx

A novel route of Ca^{2+} entry has recently been proposed in which the emptying of internal stores results in a so-called capacitative Ca^{2+}-influx. We were able to induce this form of Ca^{2+} influx, and determine the effect of CaBP, by the use of thapsigargin. Thapsigargin treatment results in a transient rise in $[Ca^{2+}]_i$ which returns to baseline over 10 min (data not shown). Perfusion of thapsigargin pre-treated cells with Ca^{2+} -free (200 μM EGTA) BSS followed by re-introduction of 1.8mM external Ca^{2+} resulted in a large sustained increase in fura-2 ratios which gradually returned towards baseline levels. When compared to HEK-p3 cultures (Fig. 16), HEK-CB12 cells exhibited a slower rate of rise in $[Ca^{2+}]_i$ (HEK-p3: 0.040±0.004 ratio units s^{-1}, N=8; HEK-CB12: 0.028±0.03 ratio units s^{-1}, N=9). No significant differences were observed in peak $[Ca^{2+}]_i$ levels.

Effect of CaBP on Cell Survival Following Excitotoxic Activation of NMDA Receptors

Given the modulatory effect of CaBP upon Ca^{2+} transients in the above experiments, studies of cell survival following NMDA channel mediated Ca^{2+} influx were carried out.

To assess the protective (or deleterious) effects of CaBP in Ca^{2+} mediated cell death, HEK-CB12 and HEK-p3 cells were transiently transfected with NR1/NR2A and

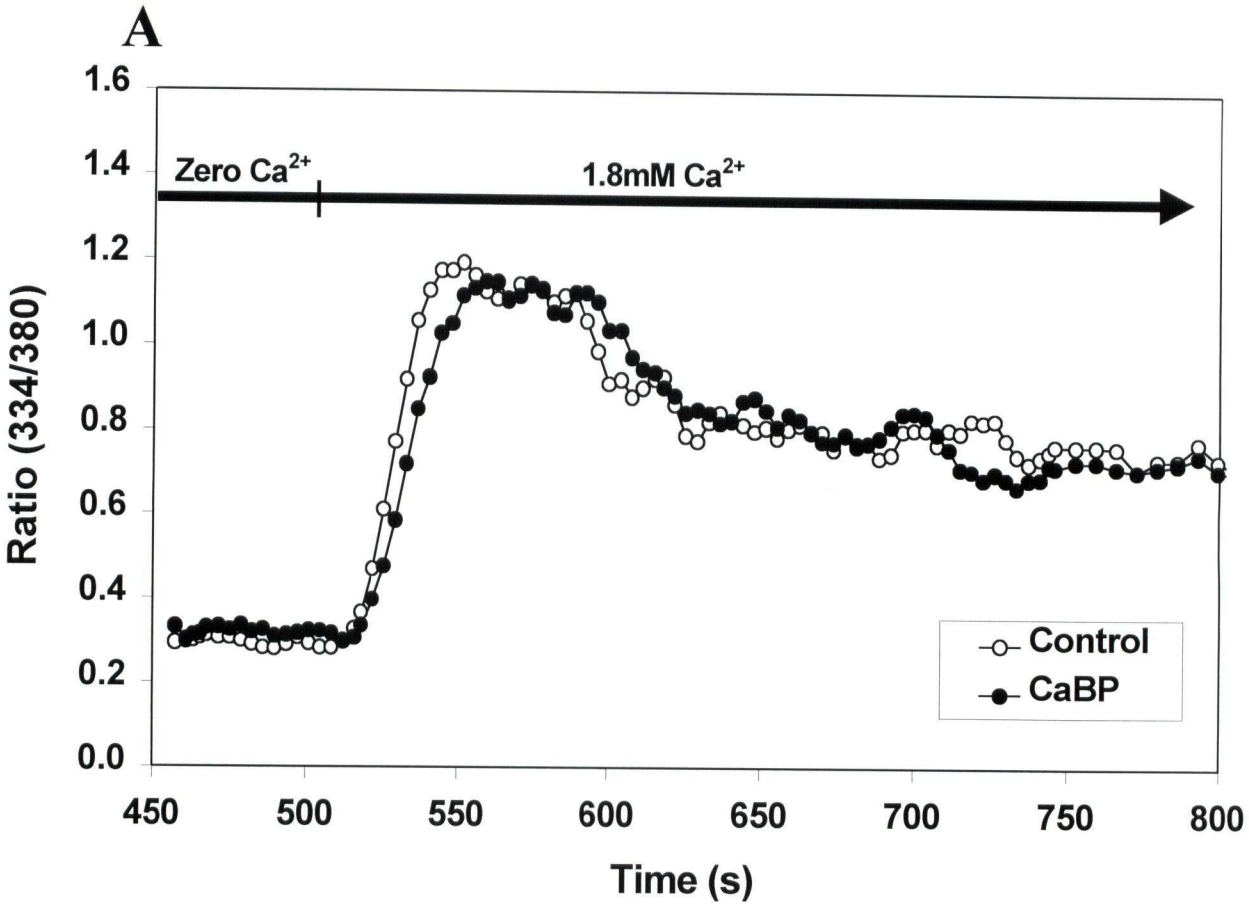
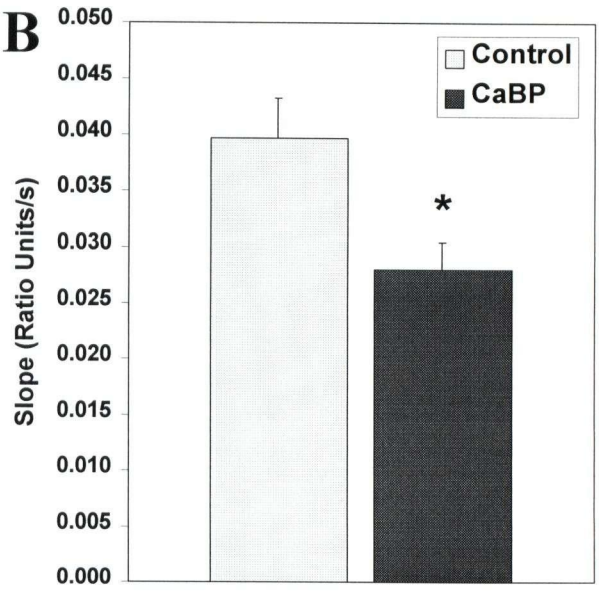


Figure 16 **Effect of CaBP upon capacitative influx of Ca^{2+} in stably transfected HEK 293 cells.** A) Representative responses of HEK-CaBP cells and HEK-p3 cells following induction of capacitative calcium influx. Cells pre-treated with thapsigargin were perfused with 200μM EGTA followed by reintroduction of 1.8mM Ca^{2+} (represented by the horizontal bar). B) Mean slopes of the rate of increase in $[Ca^{2+}]_i$ during capacitative Ca^{2+} influx. Slopes of 9 HEK-p3 cells were compared to slopes of 8 HEK-CaBP cells. Error bars indicate SEM. * $p<0.05$.



the marker pCMVβ. Transfected cultures were treated with 200 μM NMDA in BBSS for one hour (37° 5% CO_2), then returned to the original media containing 1mM APV. Control cultures were treated with 1mM APV replacing NMDA. Surviving cells were fixed 6 or 24 hours following recovery and stained for β-galactosidase. Fig. 17 illustrates the mean percentage survival of successfully transfected (as assessed by β-galactosidase staining) cells at 6 and 24 hours after treatment. At 6 hours, approximately half of the transfected cells remained, with no significant difference in survival between CaBP-CB12 and HEK-p3 cell lines (HEK-p3: 46.6±4.2 %; HEK-CB12: 44.5±5.5 %). However, at 24 hours, further significant cell death occurred in the control cultures, while the percentage of surviving HEK-CB12 cells was not significantly different from that observed at 6 hours (HEK-p3: 16.4±2.6%; HEK-CB12: 34.3±4.9%).

Discussion

Previous studies which examined the effect of CaBP on Ca^{2+} transients and neuronal survival in the face of Ca^{2+} -mediated cytotoxicity have produced contradictory results (Mattson et al., 1991; Abdel-Hamid et al., In Preparation). However, these studies examined differences between phenotypically different neurons, which could conceivably express different levels of other proteins involved in Ca^{2+} influx/homeostasis. For example, if CaBP expressing neurons also exhibited differential expression of NMDA receptors, the magnitudes of Ca^{2+} transients and therefore cell survival could be affected. In the present study therefore, we have employed a cellular

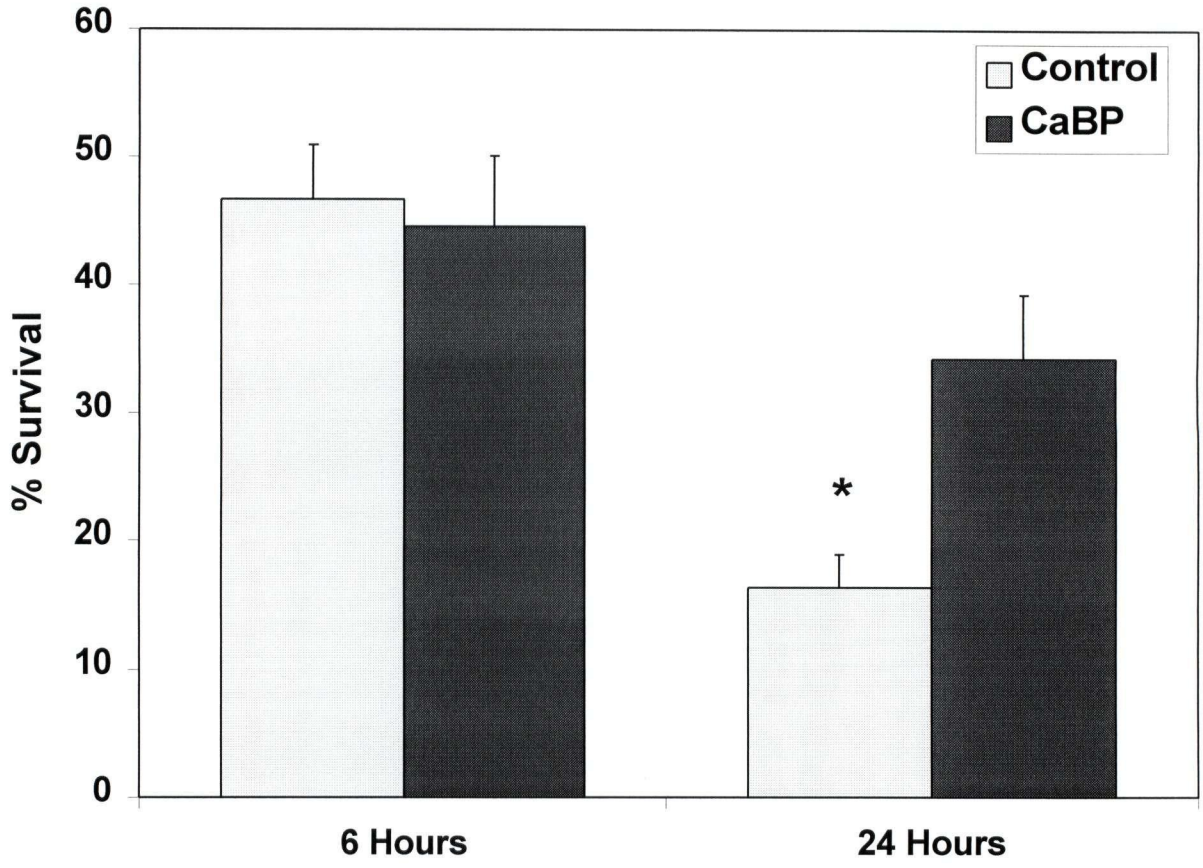


Figure 17 **Effect of CaBP upon NR1/NR2A-mediated cytotoxicity in stably-transfected HEK 293 cells.** Cell cultures were co-transfected with NR1/NR2A and pCMVβ then exposed for 1 h to 200 μM NMDA. Cells were fixed at 6 (six separate experiments) or 24 (7 separate experiments) hours post-treatment. The bars represent the mean percentage of surviving β-gal positive cells compared to APV treated controls for each cell line. Error bars indicate SEM. *$p<0.05$.

system to examine Ca^{2+} transients and cell survival, in which the only variable between control and experimental groups is the expression of CaBP.

Effect of CaBP on NR1/NR2A Mediated Ca^{2+} Transients

Prior electrophysiological studies have demonstrated that transfection of NR1/NR2A subunits results in the formation of functional NMDA receptors that are similar to those found in neurons (Raymond et al, 1996). We have examined the effect of recombinant CaBP upon whole-cell intracellular Ca^{2+} transients generated by these transfected receptors. The major effect of the presence of CaBP was a significant prolongation of the recovery to baseline values. This observation is consistent with studies of transfected or naturally occurring CaBP in other cell types (Airakisinen et al, 1997; Chard et al 1993; Lledo et al, 1992; Abdel-Hamid et al., In Preparation). However, in our experiments we did not observe a significant reduction (Chard et al 1993; Lledo et al, 1992; Airakisinen et al, 1997; Meier et al, 1998; McMahon, 1998) or increase (Abdel-Hamid et al., Unpublished Results) in peak $[Ca^{2+}]_i$ values, nor did we observe any significant effect of CaBP upon the rate of rise in $[Ca^{2+}]_i$ induced by NMDA receptor stimulation.

In considering the potential effects of CaBP, the speed of binding of Ca^{2+} to CaBP could be a critical factor, especially in relation to the speed of binding of Ca^{2+} to the indicator dye or other potential Ca^{2+}-buffering sites in the cell (see Table 1). As the concentration of Ca^{2+} ions increases within a cell, Ca^{2+} has the potential to bind to a

number of possible Ca^{2+}-buffers (including the indicator dye used, and CaBP) depending upon both the Kd of the buffer and the on-rate(s) for the high affinity binding site(s). The Kd will determine the number of available sites at any particular $[Ca^{2+}]_i$. The structure of fura-2 is based upon that of BAPTA and has a very rapid on rate for Ca^{2+} binding ($k_{on} = 7.6 \times 10^8\ M^{-1}s^{-1}$), in the order of 10 x faster than CaBP ($k_{on} = 0.77 \times 10^8\ M^{-1}s^{-1}$).

It has been previously shown that BAPTA loading of cultured hippocampal neurons results in significant alterations in depolarization or excitatory amino acid-induced Ca^{2+} transients (Abdel-Hamid and Baimbridge, 1997). Short (20-25s) exposures of BAPTA-loaded neurons to glutamate resulted in transients that had lower magnitudes, rates of rise and decay. Longer exposures (3-30 min) resulted in transients that were not lower in magnitude but still displayed reduced rates of rise and recovery. In the present study, during NMDA receptor mediated Ca^{2+} transients, it is likely that CaBP becomes saturated with Ca^{2+} during the period of continuous Ca^{2+} entry. On removal of glutamate, Ca^{2+}-entry will soon terminate and the normal Ca^{2+}-homeostatic mechanisms will begin to remove Ca^{2+} from the cell cytoplasm. For example, as the plasma membrane Ca-ATPase pumps Ca^{2+} out of the cell, a local reduction in $[Ca^{2+}]_i$ will be created near the plasma membrane that will favour the release of Ca^{2+} bound to CaBP into the cytoplasm thereby maintaining an elevated $[Ca^{2+}]_i$. Essentially CaBP is acting as a Ca^{2+}-buffer and will continue to do so until its store of bound Ca^{2+} is depleted. This may take some time since the off-rate of Ca^{2+} bound to CaBP is slow, ($K_{off} = 39.5\ s^{-1}$). This process is not unlike the mechanisms that have been proposed to account for the transcellular transport of Ca^{2+} across enterocytes in the gut (or other Ca^{2+}-

transporting epithelial cells) (Feher et al., 1992). In this process, CaBP serves as a large capacity Ca^{2+}-buffer that is in equilibrium with the prevailing $[Ca^{2+}]_i$. As $[Ca^{2+}]_i$ rises, Ca^{2+} will bind to the vacant high-affinity Ca^{2+}-binding sites of CaBP and the increase in $[Ca^{2+}]_i$ will be limited to a value close to the Kd for CaBP (513 nM) (I. Mody, Personal Communication), provided that the total net influx of Ca^{2+} does not exceed the Ca^{2+}-binding capacity of CaBP, (determined by the intracellular concentration of CaBP and the volume of the cell).

One possible alternative mechanism by which CaBP may prolong the recovery of $[Ca^{2+}]_i$ in the transiently transfected cells, is by buffering Ca^{2+} and thereby modulating the Ca^{2+}-dependent desensitization of the NMDA receptor (Legendre et al., 1993; Tong and Jahr, 1994). In effect, CaBP will increase the net influx of Ca^{2+} and thereby increase the total amount of Ca^{2+} that subsequently must be removed from the cell. Supporting this possibility are our observations that the presence of CaBP results in a significant delay in the onset of Ca^{2+}-dependent rundown of NMDA currents recorded from HEK-293 transfected with the NR1 and NR2A subunits of the NMDA receptor (Price et al., 1999).

In the experiments described above, no significant differences in either the peak Ca^{2+} or the rate of rise of $[Ca^{2+}]_i$ were observed when comparing HEK-CB12 cells with HEK-p3 cells. A possible limitation inherent in these experiments was the high degree of variability in the magnitudes of the Ca^{2+} transients, likely a result of differential transfection of the NR1/NR2A subunits. In addition, recent studies have demonstrated that fura-2 *selectively underestimates* the rise in $[Ca^{2+}]_i$ associated with the influx of Ca^{2+} through NMDA receptors (Hyrc et al., 1997; Stout and Reynolds, 1999). Given the

latter observations, it is quite possible that the increase in $[Ca^{2+}]_i$ induced by NMDA receptor stimulation, measured in these experiments with fura-2, were seriously underestimated. A very large influx of Ca^{2+} could have overwhelmed any Ca^{2+}-buffering capacity of CaBP, thus explaining the apparent lack of effect of this protein on the rate of rise and peak $[Ca^{2+}]_i$. In order to explore this possibility further, and to minimize the cell-to-cell variance in experimentally-induced changes in $[Ca^{2+}]_i$, additional experiments were conducted on the stable HEK cell lines utilizing methods which would presumably provide for a more modest and consistent stimulation of Ca^{2+} transients from cell to cell.

4-Br-A23187

Application of the ionophore 4-Br-A23187 resulted in Ca^{2+} transients which were much larger in magnitude than those observed in other Ca^{2+}-imaging experiments. CaBP clearly reduced the maximum attained $[Ca^{2+}]_i$, as well as the rates of Ca^{2+} increase and decay. This finding is significant in the light of studies which have shown that exposure to A23187 induces apoptotic cell death in neurons (Gwag et al., 1999) and that apoptotic cell death in lymphocytes is reduced in the presence of transfected CaBP (Dowd et al., 1992). Of additional interest in the report by Gwag et al (1999) is the finding that the type of cell death was dependent upon the magnitude of change in $[Ca^{2+}]_i$. High (2 μM) doses of A23187 resulted in *necrotic* cell death, whereas more moderate (250 nM) doses (with correspondingly lower fura-2 measurements) resulted in

apoptotic cell death. They suggest that this is an example of how amplitude modulation of Ca^{2+} can have differential cellular effects. Our experiments therefore suggest that CaBP may be able to similarly modulate cellular processes by reducing peak $[Ca^{2+}]_i$ responses.

ATP-Induced Release of Ca^{2+} from the ER

To assess the possible buffering effects of CaBP upon Ca^{2+} released from internal stores, Ca^{2+} transients in stable HEK cell lines were induced by exposure to ATP. ATP induces release of Ca^{2+} from the ER via the PLC-IP_3 pathway. Clearly transfected CaBP was able to attenuate the resultant rise in $[Ca^{2+}]_i$. CaBP may also influence this pathway by another mechanism. The IP_3R exhibits a Ca^{2+} feedback mechanism; at *low* $[Ca^{2+}]_i$ levels, Ca^{2+} acts synergistically with IP_3 to activate receptors and high $[Ca^{2+}]_i$ inhibits IP_3R activation (Bezprozvanny et al., 1991; Iino and Endo, 1992). (This bi-phasic sensitivity to Ca^{2+} of the IP_3R is fundamental to generation of Ca^{2+} waves which will be discussed in the following chapter.) Therefore, at low $[Ca^{2+}]_i$, CaBP may compete with IP_3 receptors for Ca^{2+}, inhibiting channel activation. At high $[Ca^{2+}]_i$, CaBP may also compete for Ca^{2+}, therefore preventing inactivation, although the Kd of CaBP suggests that it may be saturated at high $[Ca^{2+}]_i$ and therefore more effective at inhibiting channel activation at low $[Ca^{2+}]_i$. Further aspects of the effect of CaBP upon IP_3-mediated Ca^{2+} release will be discussed in Chapter 4.

NMDA-subunit-mediated and ATP-mediated Ca^{2+} release share a similar mode of change in $[Ca^{2+}]_i$: both allow external Ca^{2+} to flow down its concentration gradient into the cell, in turn creating a gradient of Ca^{2+} extending from the channels to deeper levels within the cell. To examine the effect of CaBP upon Ca^{2+} transients with different dynamic properties, we utilized a "caged" Ca^{2+} compound.

NP-EGTA

The use of the caged Ca^{2+} compound NP-EGTA allows for a global, rapid increase in $[Ca^{2+}]i$ that is independent of the activation of plasma membrane and intracellular receptors or channels (Ellis-Davies and Kaplan, 1994). The release of Ca^{2+} from NP-EGTA is extremely rapid (in the order of a few μs; Ellis-Davies and Kaplan, 1994) and would essentially be complete between the time at which exposure to UV light is terminated and the time of the initial measurement. This method also avoids the generation of steep gradients of $[Ca^{2+}]_i$ and the subsequent recovery from the increase in $[Ca^{2+}]_i$ can be observed in the absence of continued Ca^{2+} entry. Under these conditions we observed clear evidence of a Ca^{2+} buffering effect of CaBP. An initial, very rapid decline in Ca^{2+} was followed, only in the presence of CaBP, by a pronounced plateau phase and then a prolongation of the slow phase of recovery of the Ca^{2+}-transient. Similar patterns of recovery were observed in experiments employing fluo-3 and fura-2 fluorochromes. The peak Ca^{2+} response following photolysis of NP-EGTA approached 1μM, well above the estimated Kd for CaBP (513 nM I. Mody, Personal

Communication) suggesting that the protein is likely to be saturated with Ca^{2+} soon after uncaging.

In the absence of CaBP there is a prolonged fast component of the recovery phase from an increase in Ca^{2+} induced by photolysis of NP-EGTA, suggesting that, as the cell's normal Ca^{2+} homeostatic mechanisms remove excess Ca^{2+}, the component bound to the indicator dye is released relatively rapidly. Indeed, fluo-3 or fura-2 will act as a Ca^{2+}-buffer that will be in equilibrium with the prevailing $[Ca^{2+}]_i$ determined by a combination of the cell's normal Ca^{2+}-homeostatic mechanisms, (and the contribution of CaBP, if it is present). The most prominent feature of the effect of CaBP on the recovery is a pronounced plateau phase. We suggest that this plateau phase is sustained by the slow release of Ca^{2+} from the binding sites on CaBP. Essentially, as Ca^{2+} is removed from the cell it will be replaced by Ca^{2+} previously bound to CaBP until such time as all of this source of Ca^{2+} is depleted and baseline levels of $[Ca^{2+}]_i$ are restored. This mechanism is similar to that proposed to account for the role of CaBP in Ca^{2+} transport (Feher et al., 1992). Out findings lend support to this mechanism in HEK cells, as the "plateau phase" in recovery occurs at a $[Ca^{2+}]_i$ of approximately 500 nM (334/380 ratio 1.5), very close to the reported Kd (513 nM) for CaBP.

Capacitative Ca^{2+} Entry

To examine the effect of CaBP upon capacitative Ca^{2+} entry, internal stores were emptied of Ca^{2+} utilizing thapsigargin, an inhibitor of ER Ca^{2+} -ATPase (Thastrup et al.

1990). Removal and subsequent re-introduction of external Ca^{2+} resulted in a sustained rise in $[Ca^{2+}]_i$, the rate of which was decreased in the presence of CaBP (Fig. 14). The mechanism(s) responsible for this route of Ca^{2+} entry have yet to be elucidated, although it has been speculated that a so-called Ca^{2+} influx factor (CIF) is released by the ER when internal Ca^{2+} stores are depleted, and this factor in some manner induces Ca^{2+} -entry from across plasma membrane (for a review see Putney and McKay, 1999) . Whatever the mechanism, CaBP had a significant effect on the rate of $[Ca^{2+}]_i$ increase, consistent with the action of a Ca^{2+} buffer, and similar to that observed for ATP and 4-Br-A23187 stimulated Ca^{2+} transients.

Overview of Ca^{2+} Imaging Experiments

Stable HEK 293 cell lines were employed as a simple test system for the intracellular Ca^{2+} modulating potential of recombinant CaBP. Table 2 summarizes the Ca^{2+} -imaging observations made in the HEK stable cell lines. A fast, high capacity Ca^{2+} buffer would be expected to reduce the rate of $[Ca^{2+}]_i$ rise, lessen the peak $[Ca^{2+}]_i$, and prolong the recovery. In all experiments CaBP met at least one of these criteria. In experiments where ATP or 4-Br-A23187 were employed to induce Ca^{2+}-transients, all of the criteria described above were met. Of particular interest is the consistent finding that CaBP slows the recovery phase of Ca^{2+} transients regardless of the source of the Ca^{2+}. This is also in agreement with previous reports that used a wide variety of cell models and methods of inducing Ca^{2+} transients.

Table 2 Summary of Effect of Stably transfected CaBP in HEK 293 Cells

	NR1/NR2A Transfection + Glutamate	ATP	4-Br-A23187	NP-EGTA Flash Photolysis	Capacitative Ca^{2+} Influx
Rate of Rise	=	↓	↓	**NA**	↓
Peak $[Ca^{2+}]_I$	=	↓	↓	=	=
Recovery Rate	↓	↓	↓	↓	**NA**

↑ Increased relative to controls

↓ Decreased relative to controls

= No Significant difference between experimental and controls

NA No data available

The lack of a consistent effect of CaBP upon peak $[Ca^{2+}]_i$ and the rate of rise in $[Ca^{2+}]_i$ induced by the different stimuli used in the above experiments could be explained by a number of factors. First, in the case of NMDA receptor stimulation, it is likely, on the basis of recent evidence (Hyrc et al, 1997; Stout and Reynolds, 1999), that the use of fura-2 may have underestimated the rates of rise and magnitudes of the increases in $[Ca^{2+}]_i$ and that the actual values were sufficiently large to overwhelm any Ca^{2+}-buffering effect of CaBP. Second, flash photolysis of NP-EGTA results in an almost instantaneous increase in $[Ca^{2+}]_i$ increase and as such competition between the indicator dyes uses (fluo-3 and fura-2) and CaBP would be heavily in favour of binding preferentially to the former since their on-rates for Ca^{2+} are an order of magnitude faster than CaBP. Indeed, under these conditions it is likely that binding of Ca^{2+} to CaBP occurred subsequent to its release from the indicator dyes. Third, it was notable that a small but significant effect of CaBP upon the rate of rise of $[Ca^{2+}]_i$ was observed with the use of ATP, capacitative Ca^{2+}-entry, and 4-Br-A23187. In each case, the stimulus used resulted in slow rate of rise of $[Ca^{2+}]_i$ relative to that seen with NMDA or NP-EGTA.

Overall our data support a Ca^{2+}-buffering role for CaBP but also provide evidence that any Ca^{2+}-buffering effect of CaBP can be overwhelmed by very large and/or very rapid increases in $[Ca^{2+}]_i$. The consistent effect of CaBP in prolonging the rate of recovery of increases in $[Ca^{2+}]_i$, no matter the route of increase, would suggest that CaBP is more effective at buffering Ca^{2+} as it diffuses away from the regions of very high $[Ca^{2+}]_i$ that occur close to the source of Ca^{2+}-entry, i.e. close to NMDA receptor-operated channels in the plasma membrane or IP_3 and ryanodine receptors in

the endoplasmic reticulum. In this context CaBP may, as a result of its particular binding kinetics and mobility, be very effective at limiting the magnitude of the rise in $[Ca^{2+}]_i$ in areas of the cell away from the points of Ca^{2+}-entry. A similar action of CaBP has been proposed by others (Chard et al 1995; Roberts, 1994; Feher, 1983).

Excitotoxicity

To examine the effect of CaBP upon cell death induced by NMDA receptor stimulation of HEK-CB12 and HEK-p3 cells, we used a protocol modified from Raymond et al., (1996). Cells stably expressing CaBP, and transfected with the NR1/NR2A subunits of the NMDA channel, showed no difference in the degree of cell death after 6h, but a significant reduction in further cell death between 6-24h. Since greater than 50% of the cells died in the first 6h of these experiments it is likely that these cells expressed the highest levels of functional NMDA receptors, resulting in magnitudes of Ca^{2+} influx that would overwhelm any Ca^{2+}-buffering capacity of the cells, regardless of their CaBP content, and that those more moderately transfected cells survived the initial treatment, allowing detection of CaBP-enhanced survival.

The early cell death observed with excessive NMDA receptor stimulation, is likely necrotic in nature and results mostly from the influx of large amounts of sodium, chloride and water, rather than the influx of Ca^{2+}. This type of cell death has been observed both in the HEK 293 cell model system (Raymond et al., 1996), and in cultured neurons (Rothman, 1985; Olney et al. 1986; Choi, 1987), and it is not perhaps surprising that the presence or absence of CaBP has little or no effect. On the contrary, delayed cell death in models of excitotoxicity have been correlated with Ca^{2+} entry

during and after the period of stimulation, and is observed when cells are capable of recovering from the early sodium, chloride and water influx (Rothman et al 1987; Choi 1987; Randall and Thayer;1992; Manev et al 1989).

A pattern of early necrotic and delayed apoptotic cell death in neuronal cultures that is dependent upon the concentration of the agonist used, has been reported for the Ca^{2+}-ionophore, Br-A23187, (Gwag et al 1999), and for glutamate and NMDA (Ankarcrona et al 1995; Bonfoco et al 1995). In all cases high concentrations of the agonist induced necrotic cell death whereas lower concentrations resulted in apoptotic cell death. A protective effect of for CaBP against excitotoxicity has been previously shown in a variety of models of cell death (Iacopino et al., 1992; Mattson et al, 1991, Burke and Baimbridge 1993; Goodman et al; 1996; Ho et al., 1996; Meier et al., 1997, 1998; Roy et al., 1998; McMahon, 1998; Phillips et al, 1999), including two other reports that suggested a specific protection by CaBP against apoptotic cell death (Dowd et al, 1992; Diop et al 1995). Our data suggest that the failure of CaBP to protect against cell death either *in vitro* (Mockel and Fischer, 1994) or *in vivo* (Freund et al 1990) to be due to excessive stimulation leading to necrotic, rather than apoptotic death.

The results of the present studies suggest an apparent discordance between the Ca^{2+}-buffering effects of CaBP and its ability to protect cells from Ca^{2+}-mediated cell death. The lack of an effect of CaBP on peak $[Ca^{2+}]_i$ stimulated by NMDA receptor activation, and the prolongation of the recovery of Ca^{2+}-transient to baseline values, would not immediately suggest that CaBP would protect against Ca^{2+}-mediated cell death. A dissociation between measurements of $[Ca^{2+}]_i$ and cell death has been previously reported, (Michaels and Rothman, 1990) although more recent reports

suggest that this may have been due to the underreporting by the use of fura-2 of increases in $[Ca^{2+}]_i$ induced specifically by NMDA activation (Hyrc et al 1997; Stout and Reynolds 1999). However, recent reports of the role of mitochondria in cell death (Schinder et al 1996; Green and Reed, 1998; Duchen, 1999; Vergun et al, 1999; Nicholls and Budd, 2000), and in particular the observation that blockade of mitochondrial uptake can prevent neuronal cell death. (Stout et al., 1998), may provide an alternate explanation. We speculate that, through its ability to bind Ca^{2+}, CaBP could limit mitochondrial sequestration of Ca^{2+}, and hence influence cell survival following influx of excitotoxic levels of Ca^{2+}.

This possibility is of interest considering the work published on the role of CaBP in Ca^{2+} transporting tissue such as the gut, where this protein was originally discovered (Wasserman and Taylor, 1966). By comparing the relative affinities for Ca^{2+} of the plasma membrane Ca^{2+}-ATPase, CaBP and mitochondria, Rassmusen and Gustin (1978) suggested that the preferential binding of Ca^{2+} to CaBP may prevent Ca^{2+}-uptake into mitochondria during Ca^{2+}-transport; a potentially essential function considering that mitochondrial Ca^{2+}-uptake occurs at a cost to ATP generation at a time when significant amounts of ATP are required to pump out Ca^{2+} at the serosal membrane (Rassmusen and Gustin, 1978).

Summary / Conclusions

The use of stable HEK cell lines has allowed for unequivocal assessment of the effect of CaBP upon Ca^{2+} transients and NMDAR-mediated cell death. In these cell lines

diverse mechanisms were employed to transiently increase $[Ca^{2+}]_i$. CaBP was able to influence the pattern of $[Ca^{2+}]_i$ whether the Ca^{2+} was from the extracellular space (NMDAR-mediated, 4-Br-A23187, Capacitative-Ca^{2+}), intracellular stores (ATP-induced) or released globally by the caged Ca^{2+} compound NP-EGTA. Regardless of the mechanism, a Ca^{2+}-buffering effect was evident in all experiments, with the most consistent effect being a prolongation of the recovery from the induced Ca^{2+} load (Table 2). This prolongation of recovery is a consistency which runs through other CaBP-transfection studies reported in the literature. Aside from directly buffering Ca^{2+}, CaBP may also have an effect upon the mechanisms of Ca^{2+} entry, for example, by modulating Ca^{2+}-dependent inactivation (NMDAR) or activation (IP_3/Ryanodine receptors).

Evidence was also obtained to support a protective function for CaBP, based upon its Ca^{2+}-buffering capability. Specifically, it has been demonstrated that the presence of CaBP is capable of protecting cells from calcium-dependent delayed, most likely apoptotic, cell death. However, if the Ca^{2+}-buffering capacity of CaBP is overwhelmed by excessive Ca^{2+} entry, or if the stimulus also results in a large influx of sodium and chloride, the presence of CaBP fails to protect against cell death. In the latter case, the rapid cell death is most likely by necrosis. The protection conferred by CaBP against delayed, Ca^{2+}-mediated cell death, may be due to the preferential binding of Ca^{2+} to CaBP, resulting in a limitation of the available free Ca^{2+} that would otherwise be taken up by mitochondria, with the potential of triggering the release of mitochondrial apoptotic factors, leading to cell death. The ability of CaBP to protect only against delayed cell death may explain some of the contradictory results in the literature.

Chapter 4

Effect of Recombinant CaBP

on IP_3 Mediated Calcium Oscillations

Chapter Summary

1. The effects of artificial Ca^{2+} buffers and transfected CaBP on histamine-induced Ca^{2+} oscillations in HeLa cultures were examined.

2. Artificial Ca^{2+} buffers modified the oscillatory pattern of intracellular Ca^{2+} in histamine-stimulated HeLa cells. When loaded with artificial buffers, fewer cells demonstrated a distinct "baseline" oscillatory pattern.

3. Individual Ca^{2+} spikes in cells containing CaBP have a "flattened" profile; peak $[Ca^{2+}]_i$ is lowered, the rate of increase in $[Ca^{2+}]_i$ is slower and transients are prolonged.

4. A novel method for resolving Ca^{2+} waves was utilized to investigate the effect of artificial Ca^{2+} buffers and CaBP upon the propagation of IP_3-induced Ca^{2+} waves.

5. Artificial Ca^{2+} buffers and recombinant CaBP were found to retard the propagation velocity (by up to 60%) of Ca^{2+} waves in HeLa cells.

Introduction

The discussion of excitotoxicity in the previous chapter has emphasized that Ca^{2+} buffering proteins can influence the dynamics, specifically the peak $[Ca^{2+}]_i$ and the rates of rise and decay, of intracellular transients and thereby possibly affect the outcome during excitotoxic influx of Ca^{2+}. Moreover, Gwag et al (1999) have shown that two different modes of cell death can be induced, depending upon the amplitude of the rise in $[Ca^{2+}]_i$. The role of Ca^{2+} in cell death is clearly one related to pathophysiological conditions, with the exception of the type of programmed cell death that occurs, for example, during development. Under normal physiological conditions various means of Ca^{2+} signaling play a vital role in neuron function, including development and synapse formation, neurotransmitter release (Katz and Miledi, 1967), the mechanism underlying learning and memory (Bliss and Collingridge,1993), and the regulation of protein synthesis (Ghosh et al., 1994). Of increasing interest in the literature is the exploration of how Ca^{2+}, a ubiquitous second messenger, can specifically mediate such a wide variety of cellular processes. Emerging hypotheses suggest that through the regulation of spatial and temporal variations in intracellular Ca^{2+}, different signal transduction pathways can be activated (reviewed by Berridge, 1997). Intracellular signals are not only encoded by amplitudes of $[Ca^{2+}]_i$ increases, but also by patterns of Ca^{2+} influx or release, often in the form of oscillations that can be observed at a whole cell level, and Ca^{2+} waves at the sub-cellular level (Berridge, 1997). Depending upon their dynamics, such as the amplitude of a Ca^{2+}-response or the frequency of oscillations, different intracellular pathways may be activated. For example, variations in amplitude and

duration of Ca^{2+} signals in lymphocytes has been shown to mediate differential activation of transcription factors (Dolmetsch et al., 1997). It has also been demonstrated that variation in frequency of Ca^{2+} oscillations can modulate gene expression (Dolmetsch et al., 1998, Li et al., 1998). Providing a specific example in developing neurons, Gu and Spitzer (1995) have demonstrated that Ca^{2+} oscillations can influence neurite outgrowth.

Our understanding of the mechanisms leading to Ca^{2+}-oscillations and the generation of Ca^{2+} waves has been greatly advanced by studies utilizing histamine stimulation of HeLa cells (Thorn, 1995; Bootman et al., 1997). These studies have resulted in a model in which the generation of Ca^{2+} oscillations and waves are dependent on the bell-shaped sensitivity curve of Ca^{2+} on IP_3R. Further studies have suggested that endogenous Ca^{2+} buffers may play a role in modulating the information transmitted by Ca^{2+}-oscillations and Ca^{2+} waves (Clapham, 1995). In order to investigate this possibility, a HeLa cell line was generated which stably expresses CaBP (HeLa-CaBP), along with a control cell line transfected with the pCINeo vector alone (HeLa-pCINeo), to determine if the Ca^{2+} buffering properties of CaBP demonstrated in other cell types could affect the dynamics of Ca^{2+} oscillations and waves induced by the action of histamine.

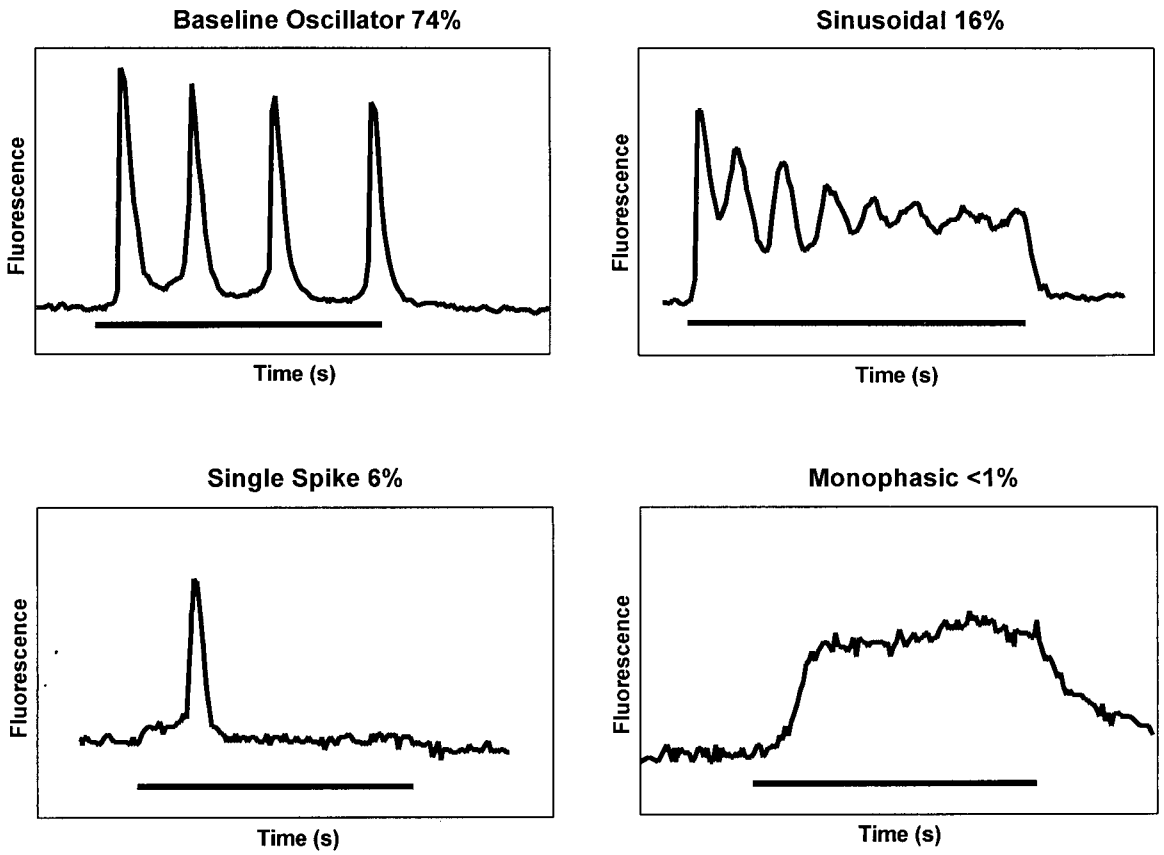
Results

Effects of Indicator Dyes on Ca^{2+} Oscillations

In initial experiments with HeLa cells it was determined that the loading concentration of the Ca^{2+} indicator fluo-3 significantly influenced the Ca^{2+}-oscillatory behaviour of HeLa cells. When loaded with 3.1 μM fluo-3 for 60 min at room temperature, very few cells displayed oscillations in which the level of $[Ca^{2+}]_i$ returned to near-baseline levels prior to the initiation of the subsequent Ca^{2+} spike (data not shown). It was determined empirically that reducing the loading concentration of fluo-3 resulted in more defined Ca^{2+} spikes. Therefore in all subsequent experiments cells were loaded with 0.75 μM fluo-3 for 60 min, both in studies of fields of oscillating cells and in high magnification studies of Ca^{2+} waves in individual cells.

In a typical field of cells, it was observed that almost all cells displayed a change in $[Ca^{2+}]_i$ in response to superfusion with 1μM histamine. However, in agreement with Missiaen et al., (1993), we also observed a variety of different responses from cell to cell. Therefore, the type of Ca^{2+} oscillations were categorized as shown in Fig. 18, following the nomenclature of Missiaen et al. (1993). The majority of cells responded with slow (~45s duration) Ca^{2+} oscillations with each successive peak typically being of lower magnitude than its predecessor.

In additional experiments using fura-2, we observed that the influence of this indicator dye upon the type of response of HeLa cells to histamine, was even more pronounced than that observed with fluo-3. This problem was alleviated (but not to the



No response or other: 4%

Figure 18 **Classification of Calcium Oscillatory Types in HeLa cells.** Representative traces of individual HeLa cells loaded with fluo-3 and exposed to continuous perfusion of 1 μM histamine at room temperature. Indicated percentages are from a population of 196 individual cells. The horizontal bar indicates the 300s 1μM histamine perfusion.

extent seen with the use of lower loading concentrations of fluo-3) by reducing the loading concentration of fura-2 to the minimum possible levels for detection on our imaging system. Typically the use of fura-2 resulted in a greated proportion of cells exhibiting a "sinusoidal" oscillatory pattern (see Fig. 18).

In view of our observed differences between responses using either fluo-3 or fura-2, initial experiments examining the effect of artificial Ca^{2+} buffers and CaBP on Ca^{2+} oscillatory patterns were performed utilizing fluo-3. However, some experiments were also performed using cells minimally loaded with fura-2, particularly when we considered it important to attempt to quantify the observed changes in $[Ca^{2+}]_i$.

Effect of Artificial Calcium Buffers on the Oscillatory Behavior of HeLa Cells

HeLa cell cultures were loaded with fluo-3 alone or combinations of fluo-3 and cell-permeant artificial Ca^{2+} buffers, then superfused for 300s with 1 μM histamine. The intensity of fluorescence from individual cells was determined on an Attofluor imaging system (63x Objective) by placing a single "region of interest" over each cell in the field of view.

When compared to HeLa cells loaded with fluo-3 alone, the effect of loading cells with 2.5 μM EGTA-AM was a small increase in the percentage of cells demonstrating a more sinusoidal response at the expense of a reduction in those having an oscillatory response (Fig. 19). Increasing the loading concentration of EGTA-AM to

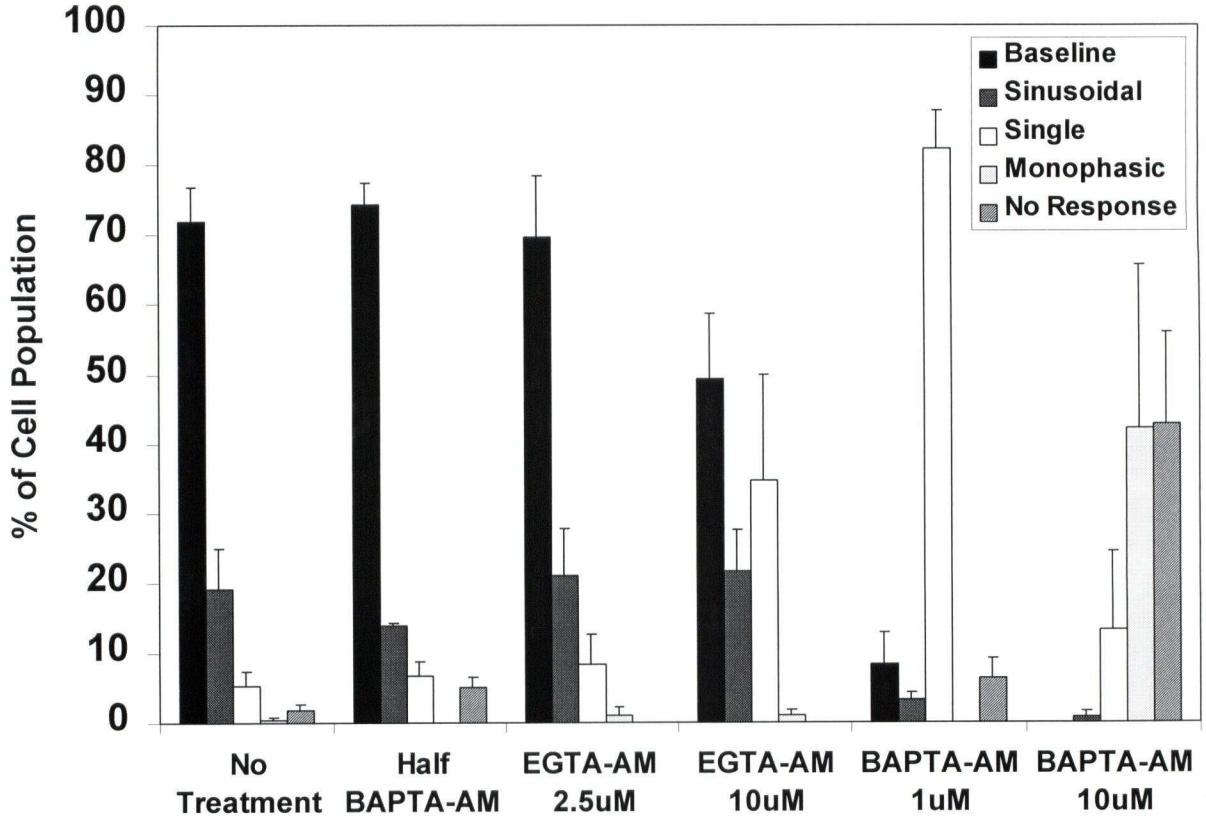


Figure 19 **Effect of Calcium buffers on Oscillatory Behaviour of HeLA Cells**. HeLa cells co-loaded with fluo-3 and the indicated buffer were exposed to 300s perfusions of 1 μM histamine at room temperature. The pattern of calcium oscillation was classified according to Figure 18. Bars indicate the proportion of cells displaying the indicated oscillatory pattern. Total cells counted for each experiment were as follows: No Treatment: N=5, 196 cells total; Half-BAPTA: N=3, 106 cells total; 2.5 μM EGTA: N=3, 65 cells total; 10 μM EGTA: N=3, 102 cells total; 1 μM BAPTA: N=3, 88 cells total; 10 μM BAPTA: N=3, 149 cells total.

10 μM had the effect of reducing the total number of baseline oscillators and increasing the percentage of cells which responded with a single Ca^{2+} spike. Cells loaded with 1 μM BAPTA-AM displayed a significantly different pattern of Ca^{2+} response to 1 μM histamine (Fig. 19), with the majority of cells responding with a single Ca^{2+} transient. A loading concentration of 10 μM BAPTA-AM completely abolished repetitive oscillations and most cells exhibited a "monophasic" Ca^{2+} response to histamine. In addition, 43% of cells loaded with this concentration of BAPTA failed to show any detectable increase in fluo-3 fluorescence.

Effect Of CaBP on Calcium Oscillations

Continuous superfusion of HeLa-CaBP cells with 1 μM histamine produced oscillatory patterns that were not significantly different from HeLa-pCINeo cells (Fig. 20). An additional analysis of the frequency of Ca^{2+} oscillations was carried out by counting the number of peaks in $[Ca^{2+}]_i$ over 400s and this also revealed no significant differences between HeLa-pCINeo (0.93±0.04 oscillations/min, N=35) and HeLa-CaBP cells (0.95±0.03 oscillations/min, N=66). Unfortunately, the wide variety of oscillatory patterns and changing frequencies over time precluded any definitive conclusions as to the effect of CaBP on oscillation frequency.

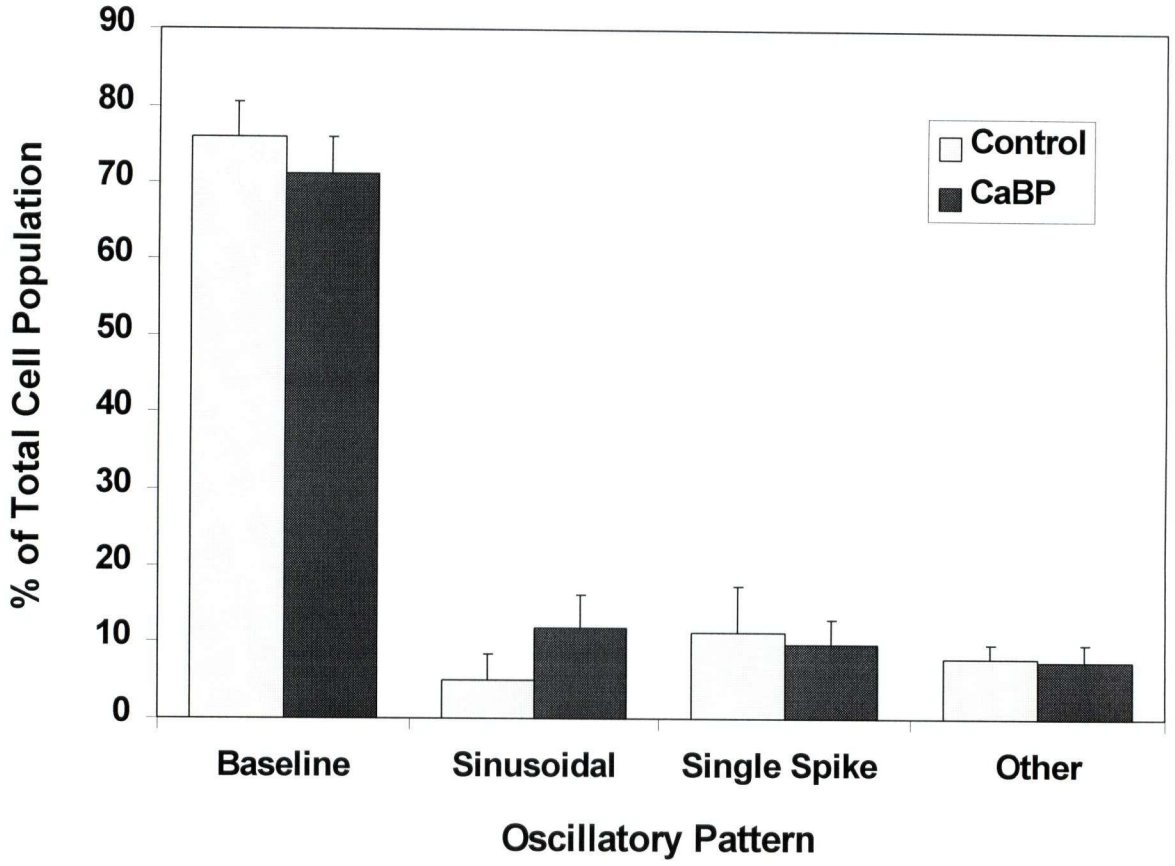


Figure 20 **Effect of recombinant CaBP on calcium oscillatory pattern in stable HeLa cell lines.** HeLa cells stably transfected with CaBP or control vector were loaded with fluo-3 and the fluorescence response of individual cells recorded during a 300s perfusion with 1 μM histamine at room temperature. The pattern of calcium oscillation was classified according to figure 18. Bars indicate the proportion of cells (HeLa-pCINeo N=4; 143 cells total; HeLa-CaBP: N=5, 154 cells total) which displayed the indicated oscillatory pattern. Error bars indicate SEM.

When individual Ca^{2+} oscillations were examined, it was found that the mean time for a complete oscillation in HeLa-CaBP cells was significantly prolonged (45.31±1.94 s, N=32), when compared to that in HeLa-pCINeo cells (39.54±1.6 s, N=33 $p<0.05$) (Fig. 21). This prolongation effect was confirmed through analysis of span times of normalized fluorescence values (25%-span: HeLa-pCINeo: 19.14±0.74 s, N=33; HeLa-CaBP: 23.81±1.53 s, N=32; 50%-span: HeLa-pCINeo:11.81±0.64 s HeLa-CaBP:14.98±1.13 s, $p<0.05$). In addition, high-speed imaging studies, carried out in conjunction with wave resolution experiments described in the following section, revealed that, when treated with 100μM histamine, the rate of increase in $[Ca^{2+}]_i$ (expressed as $\Delta F/F\ s^{-1}$) was significantly reduced from 1.362± 0.164 $\Delta F/F\ s^{-1}$, N=7 in HeLa-pCINeo cells to 0.914±0.096 $\Delta F/F\ s^{-1}$, N=19 in HeLa-CaBP cells (Fig. 22).

In order to assess any effect of CaBP upon the magnitude of $[Ca^{2+}]_i$ responses, an analysis of HeLa cell responses was also carried out using cells minimally loaded with the ratiometric dye fura-2. In the majority of both HeLa-CaBP and HeLa-pCINeo cells, a "sinusoidal" pattern of Ca^{2+} oscillation was observed and the elevated levels of $[Ca^{2+}]_i$ persisted until the histamine was removed (Fig. 23A). In order to assess the role of external Ca^{2+} in the response of HeLa cells to histamine, these experiments were repeated with 200μM EGTA and no added Ca^{2+} in the perfusate. Under these conditions, $[Ca^{2+}]_i$ returned to baseline levels prior to the washout of the histamine (Fig. 23B). The peak $[Ca^{2+}]_i$ appeared to be unaffected: maximum ratios (Fig. 24A) were not significantly different from cells superfused with media containing 1.8mM Ca^{2+} and there was still a significant difference between HeLa-pCINeo and HeLa-CaBP cells

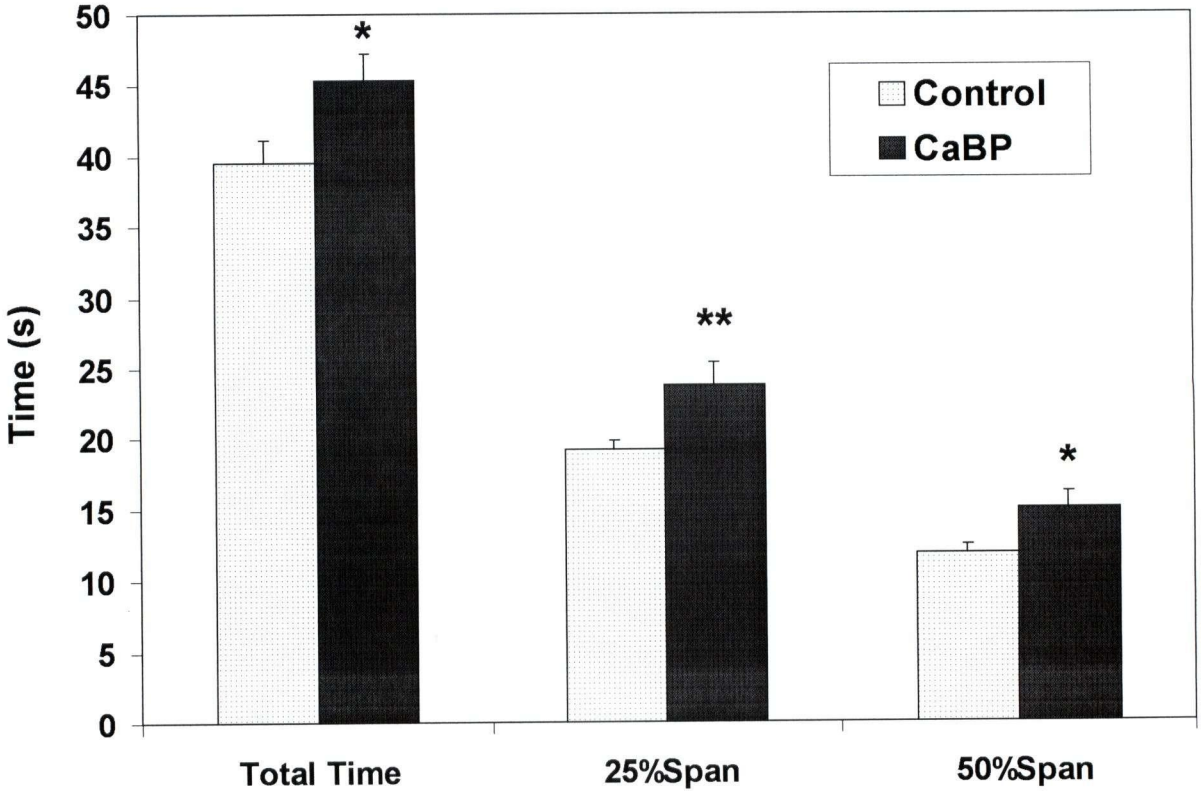


Figure 21 **Influence of CaBP upon individual calcium transients in oscillating stable HeLa cells.** Single calcium transients in the oscillatory pattern of HeLa-pCINeo and HeLa-CaBP cells exposed to 1μM histamine at room temperature were analyzed. The time of the total individual oscillation was determined as was the span (time) at 25% and 50% of the maximum response. Error bars indicate SEM. (* $p<0.05$; ** $p<0.01$)

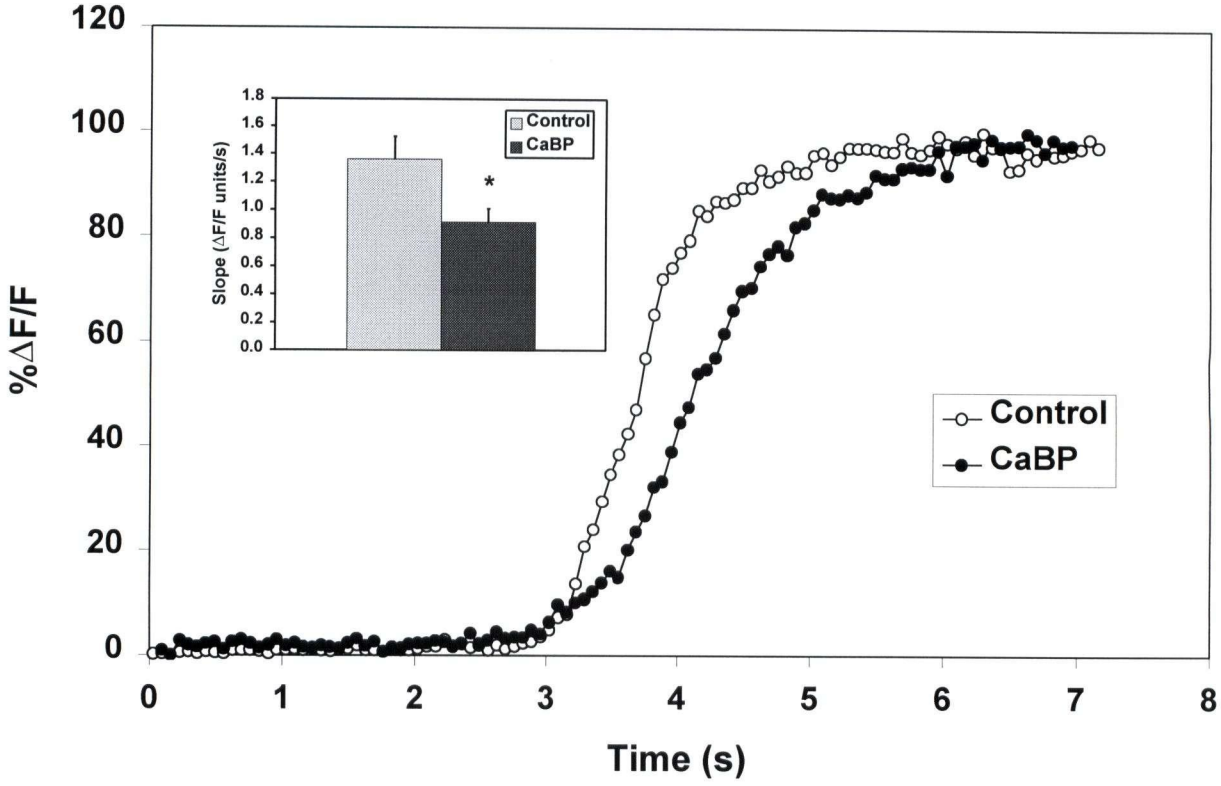


Figure 22 **The effect of CaBP upon rate of Ca^{2+} increase in stable HeLa cells treated with 100μM histamine.** Representative traces of two stably transfected HeLa cells imaged at 15 frames per second following addition of 100μM histamine at RT. Fluorescence data was converted to % ΔF/F and the rate of increase was determined between 25% and 50% of the maximum fluorescence measured. The inset chart displays the pooled data of 7 HeLa-pCINeo and 33 HeLa-CaBP cells. Error bars indicate SEM. (*$p<0.05$)

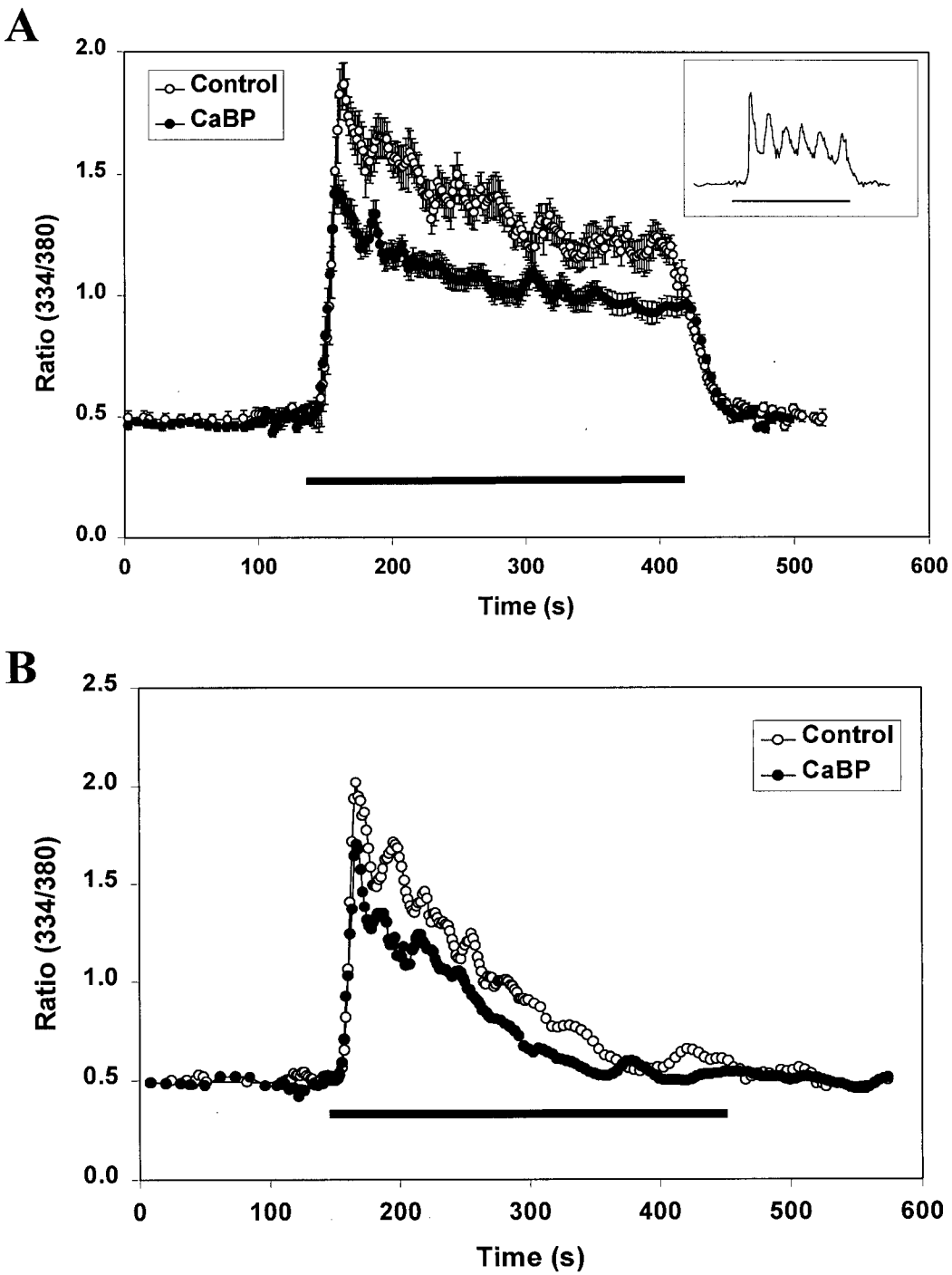


Figure 23 **Effect of CaBP on response of HeLa cells loaded with fura-2 and treated with 1μM histamine. (A)** Mean $[Ca^{2+}]_i$ response of HeLa-pCINeo (N=32) and HeLa-CaBP (N=42) cells treated for 400s with 1μM histamine RT. Bar indicates treatment period. Inset: Single cell (HeLa-pCINeo) oscillator example. **(B)** Mean $[Ca^{2+}]_i$ responses of HeLa-pCINeo (N=34) and HeLa-CaBP (N=42) cells treated for 400s with 1μM histamine in the absence of external Ca^{2+} (BSS + 200 μM EGTA; No added Ca^{2+})

(HeLa-pCINeo: 2.6±0.11 N=32; HeLa-CaBP: 2.0±0.08 N=42; Ca^{2+}-calibrated values: HeLa-pCINeo: 710 nM ; HeLa-CaBP: 472 nM). However, the average maximum 334/380 fluorescence ratio (Fig. 24A) was significantly less in CaBP-containing cells (HeLa-pCINeo: 2.6±0.13, N=32; HeLa-CaBP: 1.9±0.08, N=42; Ca^{2+}-calibrated values: HeLa-pCINeo: 710 nM ; HeLa-CaBP: 437 nM).

Figure 24B summarizes the effect of CaBP upon the relative total intracellular Ca^{2+} in histamine treated HeLa cells as determined by the integrated areas under the graphs of fluorescence ratio vs time. The presence of CaBP resulted in a significant reduction in average integrated area (HeLa-pCINeo: 455.4±16.06, N=32; HeLa-CaBP: 361.8±13.36, N=42). In similar experiments where Ca^{2+} was removed from the bathing media, a significant reduction in integrated area (HeLa-pCINeo: 294±10.08, N=34; HeLa-CaBP: 235±8.20, N=42) was also observed (Fig. 24B). The sinusoidal oscillatory pattern exhibited by the majority of fura-2 loaded cells prohibited an analysis of span times and recovery to baseline.

Calcium Wave Propagation in Stable HeLa Cell Lines

The above experiments described the changes of Ca^{2+} observed in entire cells in response to histamine. In order to resolve properties of the Ca^{2+} waves in HeLa cells, a technique employing a standard epifluorescence Ca^{2+}-imaging apparatus was developed (other reports of Ca^{2+} wave velocity have typically employed confocal laser scanning

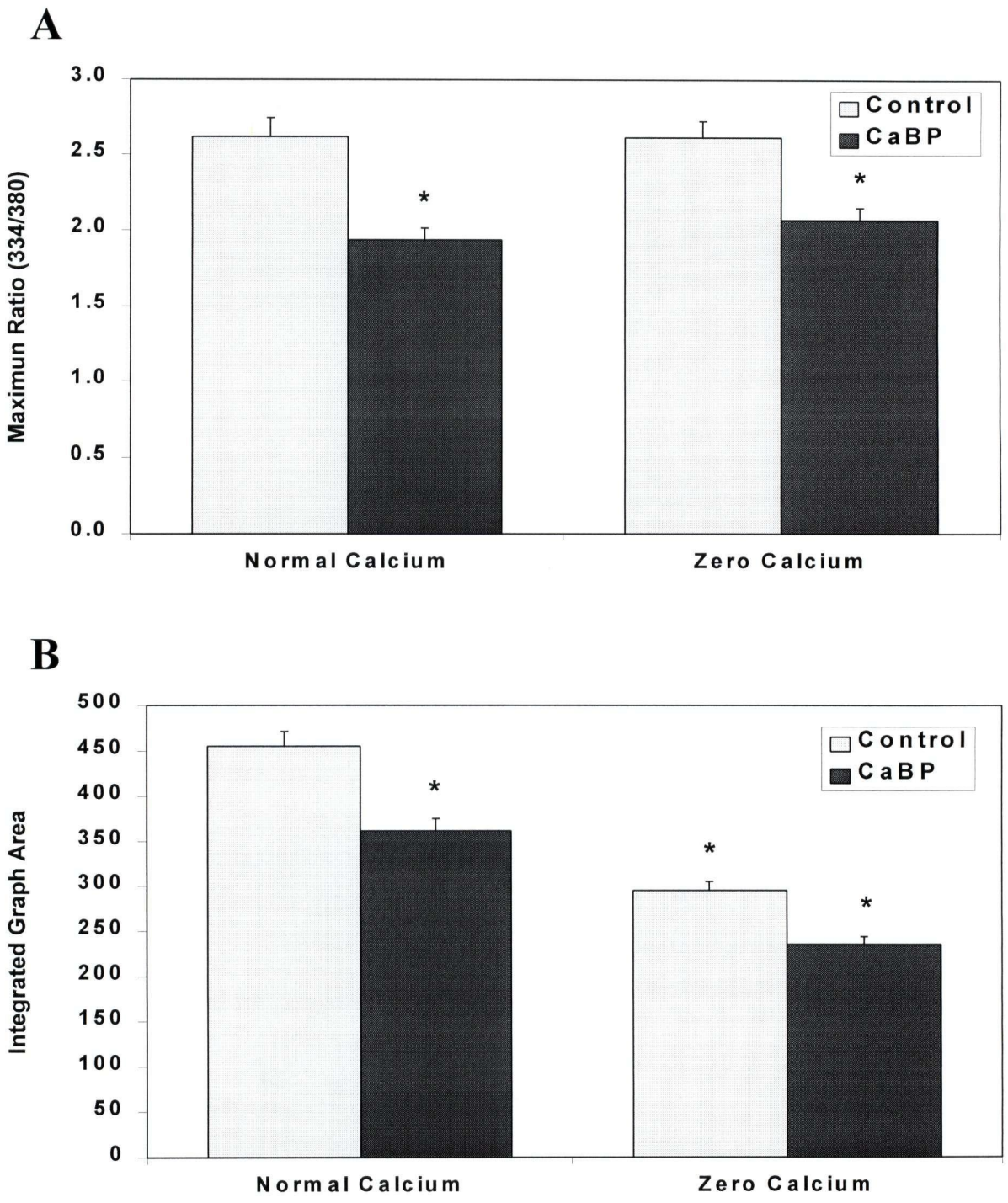


Figure 24 **Effect of recombinant CaBP upon Ca^{2+} influx in fura-2 loaded HeLa cells treated with histamine. (A)** Comparison of peak fura-2 ratios of HeLa-pCINeo (N=34) and HeLa-CaBP (N=42) cell lines loaded with fura-2 and treated with 1μM histamine in the presence of 1.8mM calcium or absence of external Ca^{2+} . **(B)** Integrated area under traces of fluorescence response of fura-2 loaded HeLa-pCINeo (N=34) and HeLa-CaBP (N=42) to 1μM histamine in the presence or absence of external Ca^{2+} . Error bars indicate SEM. * =$p<0.05$.

microscopy). A very similar technique for measuring Ca^{2+}-wave velocity was subsequently described by Wang et al (1997). Following superfusion of 100 μM histamine, a region of interest, (1 pixel wide and up to 250 pixels or 75μm long) was placed over single HeLa cells and scanned at 15 frames per second under high magnification (100x objective). The fluorescence intensity of each pixel along this strip was quantified. Averaged fluorescence values from these scans were used to determine global changes in cell $[Ca^{2+}]_i$ (see Fig. 22), while an analysis of individual pixels was used to resolve the "front" of Ca^{2+} waves. The percentage change in ΔF/F values were calculated for each pixel along the scanned line and the time at which each point surpassed a 50% increase in ΔF/F was plotted against its position along the scanned line. From this plot the approximate origin of the Ca^{2+} wave could be determined along with the propagation velocity of the wave. We observed, as have other authors (Bootman et al.,1997), that Ca^{2+} waves with single initiation sites were more reproducibly generated with the application of 100 μM histamine. In the example shown in Fig. 25, a single initiation site is evident. Cells did not appear to have a common region where waves were initiated, as both sites near the nucleus and in the cell periphery were observed, resulting in wave propagation away from or towards the center of the cell respectively. Successive histamine responses in the same cell resulted in wave initiation from the same region (data not shown) and some cells displayed more than one initiation site (Fig. 26) or an indistinct Ca^{2+}-wave. These were excluded from the detailed analysis of wave velocity as described below.

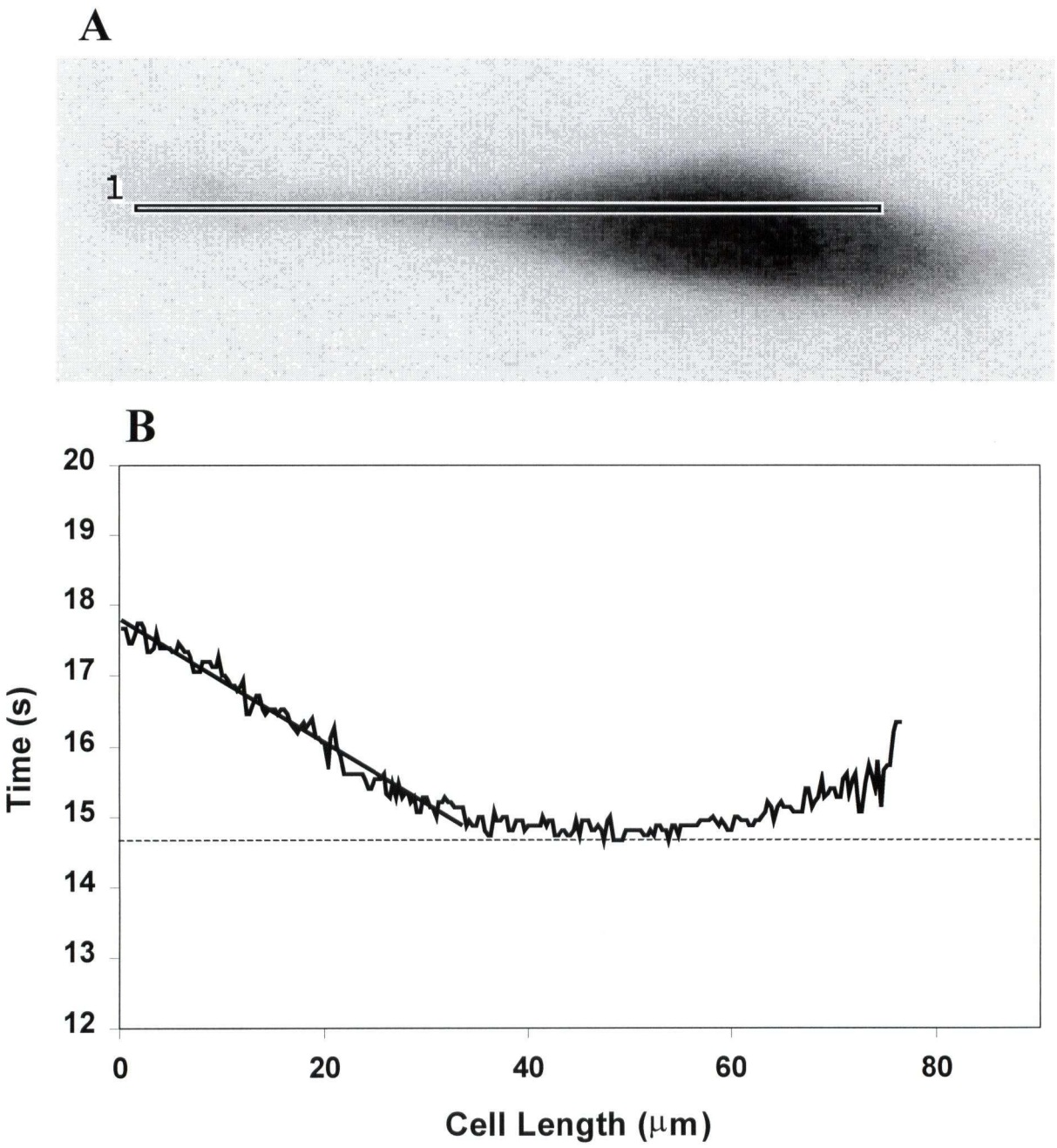
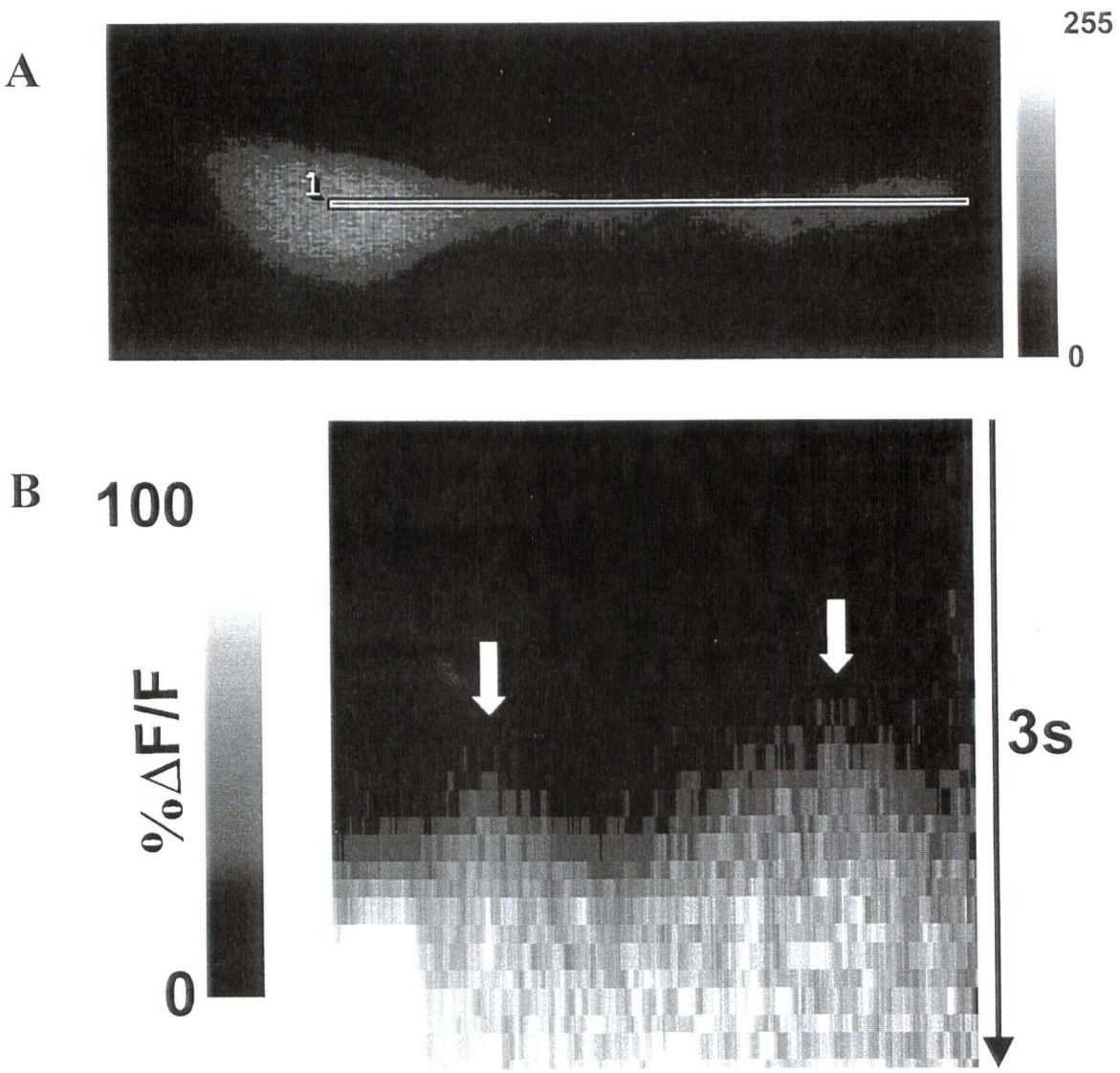


Figure 25 **Example determination of wave velocity for an individual HeLa cell treated with 100μM histamine.** (A). Digitized fluorescence image (inverted greyscale for clarity) of a single HeLa Cell loaded with fluo-3. The horizontal bar indicates the region scanned for changes in fluorescence. The horizontal dimension is preserved in the X-axis of the graph in B. **(B)** Plot of calcium wave propagation in a fluo-3 loaded HeLa cell. The plot indicates the time at which that particular point along the linescan reaches 50% ΔF/F. The line of best fit (solid line) was used to determine wave velocity. The dotted line represents the minimum time required for a 50% ΔF/F at the initiation site.

Figure 26 **Resolution of two calcium waves, initiated at distinct sites in an individual HeLa cell treated with 100μM histamine.** (**A**). Pseudo-colour digitized fluorescence image of a single HeLa Cell loaded with Fluo-3. The region indicated in the micrograph (1) was scanned for changes in fluorescence. The pseudo-colour scale is indicated on the left. The horizontal dimension of the micrograph is preserved in B and C. **(B)** Pseudo-colour composite of linescans of a single HeLa cell treated with 100μM histamine. %ΔF/F values were calculated from fluorescence images obtained from the linescan depicted in panel A. Pseudo-colouring was applied to each image as indicated by the bar left of the image, and images were stacked vertically. Arrows indicate the regions of calcium wave initiation. **(C)** Plot of 50% threshold of linescan. Individual points along the line represent the time at which the pixel at the position indicated on the x-axis surpassed the 50%ΔF/F threshold. Arrows indicate regions of wave initiation.


A
255
1
0
B
100
%ΔF/F
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3s
C
6.6
6.5
6.4
6.3
6.2
6.1
6.0
Time (s)
50% Threshold
0
20
40
60
80
Cell Length (μm)

Effect of Artificial Calcium Buffers and CaBP upon Calcium Wave Propagation

The velocity of Ca^{2+} waves were determined in HeLa cells loaded with Ca^{2+} buffers (see Methods section). In addition, the velocities of Ca^{2+} waves in HeLa-pCINeo controls were compared to those in HeLa-CaBP cells. Controls cells had an average velocity of 81.4±10.5 μm s^{-1} (N=10) whereas loading with 2.5μM EGTA-AM, 1μM BAPTA-AM or 10μM BAPTA-AM significantly reduced the average velocity to 50.2±5.3 μm · s^{-1} (N=7), 38.2±6.1 μm · s^{-1} (N=5), and 22.0±4.6 μm s^{-1} (N=5), respectively (Fig. 27). When wave velocity determination was carried out in HeLa-CaBP cells, a reduction in velocity to 49.5±6.66 μm · s^{-1} (N=12), similar to the effect of 2.5 μM EGTA was observed.

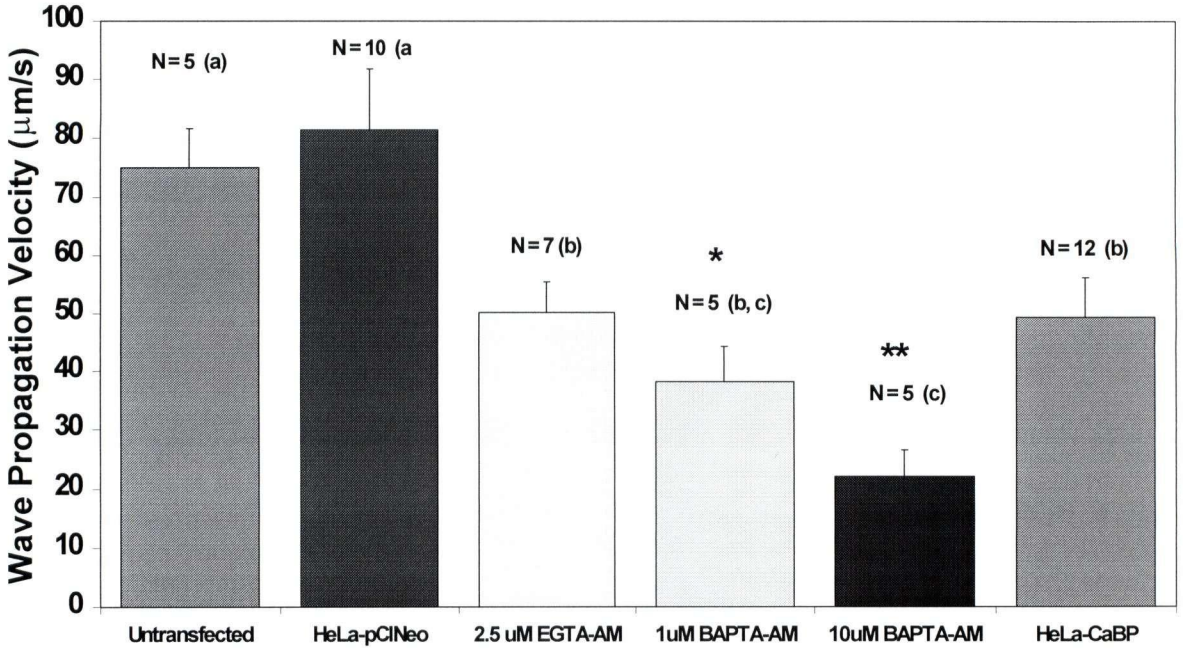

Figure 27 **Summary of resolved calcium wave velocities in HeLa cells.** Bars indicate mean calculated velocity of calcium waves in transfected (HeLa-p3 and HeLa-CaBP) HeLa cells and untransfected HeLa cells loaded with the indicated calcium buffer. Indicated significant differences are in comparison to the appropriate control * $p<0.05$ ** $p<0.01$; *** $p<0.001$. The mean value of bars indicated by common letter code(s) are not significantly different from each other.

Discussion

It has been recognized for some time that second messenger signaling by Ca^{2+} is not solely enacted through simple increases or decreases in $[Ca^{2+}]_i$. Although information can be encoded by the amplitude of the increase in $[Ca^{2+}]_i$, oscillatory frequency has also been shown to mediate numerous cellular processes (for a review see Berridge, 1997). It has therefore been proposed that proteins which bind Ca^{2+} may modulate this signal and hence influence the transduction of information in cells (Clapham, 1995). The availability of HeLa cells stably transfected with CaBP, enabled us to examine the effects of CaBP upon histamine-induced Ca^{2+}-signalling in this cell line and to compare these effects with that of artificial Ca^{2+}-buffers.

Calcium Oscillations in HeLa Cells

The oscillatory behaviour of fluo-3 loaded HeLa cells observed in these experiments were similar to those observed by other authors (Missiaen et al, 1993). The majority of fluo-3 loaded cells demonstrated repetitive Ca^{2+} transients in which Ca^{2+} levels returned to baseline prior to the onset of the next spike. This oscillatory behaviour was dependent upon refilling of internal stores by external Ca^{2+} (Fig. 23B). Ca^{2+} oscillations in response to histamine are a result of the bi-phasic sensitivity curve of the IP_3R. Bezprozvanny et al. (1991) demonstrated that the IP_3R open-probability increases

with increasing $[Ca^{2+}]_i$ to a maximum at ~250 nM $[Ca^{2+}]_i$. Increasing $[Ca^{2+}]_i$ above this level results in a progressively lower open-probability. With this Ca^{2+}-sensitivity in mind, the initial impetus for these experiments was to determine whether or not Ca^{2+} buffers and/or CaBP might have an effect upon the frequency of Ca^{2+} oscillations in HeLa cells. However our initial examination of oscillatory behaviour of populations of wild-type HeLa cells revealed that cell-to-cell heterogeneity of oscillatory frequency, and changing frequencies in individual cells, might preclude any definitive examination of frequency modulation in this cell type. Nonetheless, modulation of Ca^{2+} oscillatory behaviour was detected by an examination of the relative proportion of the various oscillatory patterns.

Effects of Indicator Dyes

Our observation that the oscillatory behaviour is significantly affected by the loading duration and concentration of fluo-3 suggests that the Ca^{2+}-buffering effect of this indicator dye may interfere with the natural physiological response of HeLa cells to histamine. Indeed, this is not entirely unexpected since the properties of fluo-3 (see Table 1) are similar to its parent compound, BAPTA. In the particular case of fluo-3 however, an alternative explanation is possible since it has been shown that fluo-3 can act as a competitive antagonist of IP_3 receptors (Richardson and Taylor, 1993). In our experiments, reducing the loading concentration of fluo-3 resulted in more consistent oscillatory responses. This observation further emphasizes the importance of minimal

loading with Ca^{2+} indicators and the need for caution in interpretation of experiments utilizing these compounds.

Ca^{2+} Diffusion, Fixed and Mobile Ca^{2+} Buffers

Given that generation of Ca^{2+} waves is dependent upon Ca^{2+} released from the ER, and that the diffusion of this Ca^{2+} is responsible for the propagation of Ca^{2+} waves, it becomes important to understand how mobile buffers, such as BATA, EGTA and CaBP, influence Ca^{2+} diffusion within living cells. It has been shown in many cell types, including gonadothrophs, GH_3 cells, and neurons, that 99% of the Ca^{2+} which enters the cell is rapidly bound by endogenous, immobile Ca^{2+} buffers (Tse et al., 1994; Lledo et al., 1992; Fleet et al., 1998; Fierro and Llano, 1996; Palecek et al., 1999). The remaining 1% is free ionic Ca^{2+} which can interact with mobile Ca^{2+} indicators and/or Ca^{2+} buffers, which in turn may modulate Ca^{2+} oscillations and waves.

Ca^{2+} Oscillations and Artificial Ca^{2+} Buffers in HeLa Cells

To characterize the effect of artificial Ca^{2+} buffers on Ca^{2+} oscillations, HeLa cells minimally loaded with fluo-3 were also loaded with either EGTA or BAPTA. In experiments where HeLa cells were loaded with low (2.5 μM) concentrations of EGTA-

AM, "baseline" Ca^{2+} oscillations gave way to more sinusoidal oscillatory patterns, which in turn, were replaced by single Ca^{2+} transient that returned to baseline when the loading concentration was increased to 10 μM. In comparison, BAPTA abolished histamine-induced Ca^{2+} oscillations at all tested loading concentrations. At loading concentrations of 1 μM, HeLa cells predominately responded to histamine with a single Ca^{2+} spike, whereas cells loaded with 10 μM BAPTA responded with a delayed, slow, monophasic rise in $[Ca^{2+}]_i$ followed by a slow return to basal levels upon wash-out of histamine or failed to respond.

Histamine-induced Ca^{2+} oscillations in HeLa cells are a product of the bi-phasic sensitivity of the IP_3R to Ca^{2+} (Bezprozvanny et al., 1991). At low levels (< 250nM), increasing intracellular Ca^{2+} acts synergistically with IP_3 to induce Ca^{2+} release from the ER. This positive feedback system is responsible for the rising phase in $[Ca^{2+}]_i$ oscillations. At high intracellular concentrations, Ca^{2+} inhibits the action of IP_3 on its receptor, resulting in a return to near basal levels of $[Ca^{2+}]_i$ as Ca^{2+} is cleared by Ca^{2+}-ATPases or by other intracellular Ca^{2+} homeostatic mechanisms. Ca^{2+} waves are initiated from so-called "hot-spots" in the cell, which are likely clusters of IP_3 receptors (Bootman et al.,1997). The effect of this clustering is the overlapping of microdomains of released Ca^{2+}, resulting in an increased local $[Ca^{2+}]_i$ and an increased open-probability of adjacent receptors. This Ca^{2+}-mediated recruitment of adjacent receptors is thought to be the underlying mechanism of Ca^{2+} oscillation initiation and Ca^{2+} wave propagation.

In attempting to predict the effect of a Ca^{2+} buffer upon IP_3-mediated Ca^{2+} release both the speed of binding and the level at which $[Ca^{2+}]_i$ would be buffered (i.e. the Kd of the buffer) must be taken into consideration. A buffer that binds Ca^{2+}

significantly slower than the IP_3R itself is unlikely to have a significant effect. An appropriate analogy would be the fact that, with respect to neurotransmitter release in the squid giant axon synapse, the fast Ca^{2+}-buffer BAPTA significantly reduces release whereas the slow Ca^{2+}-buffer, EGTA has little effect (Adler et al., 1991). With respect to the Kd, a buffer with a *low* Kd (~ 250 nM or less) may maintain $[Ca^{2+}]_i$, in the range where it promotes IP_3-mediated Ca^{2+}-release whereas one with a *high* Kd (> 500 nM) may maintain $[Ca^{2+}]_i$ in the range where it inhibits IP_3-mediated Ca^{2+}-release. Based upon the known properties of BAPTA (Kd=0.50 μM; Pethig et al., 1989, k_{on}= $6x10^8$ M^{-1} s^{-1}; Tsien, 1980, see Table 1) and the ranges of $[Ca^{2+}]_i$ required for activation and inhibition of IP_3-mediated Ca^{2+}-release it is likely that BAPTA may initially inhibit IP_3R activation by competing with adjacent receptors in "hot spots" for released Ca^{2+} (or prevent channel activation altogether if the buffer concentration is high enough). Following activation however, BAPTA may enhance additional Ca^{2+} release and prolong elevation of $[Ca^{2+}]_i$ by buffering $[Ca^{2+}]_i$ near the concentration where Ca^{2+} acts synergistically with IP_3 to open the IP_3R (~250 nM; Bezprozvanny et al, 1991). This hypothesized action of BAPTA is supported by the results presented in this chapter in that HeLa cells loaded with BAPTA either fail to respond to histamine, or respond with a slow-onset, prolonged, monophasic rise in $[Ca^{2+}]_i$ which slowly returns to basal levels upon washout of histamine. It must also be considered that the observed effect of BAPTA could be mediated by BAPTA directly inhibiting the IP_3 channel (as described above for fluo-3).

It should be noted also that, as with the case of fluo-3-AM and fura-2-AM, the final intracellular concentration of the AM-derivatives of artificial Ca^{2+} buffers is

unknown. Due to the mechanism of loading -AM derivatives, it is possible for the cells to accumulate the buffer to levels well above that of the loading solution. For example, it has been estimated that intracellular concentration of -AM derivatives of BAPTA analogs may reach 20 to 40 times the loading concentration (Wang and Thompson, 1995).

The dramatic difference in effects of EGTA and BAPTA are interesting in that the two buffers have a similar Kd (see Table 1, Introduction). However, there is a 300-fold difference in the compounds on-rates (K_{on}) (BAPTA: $600x10^6 M^{-1} s^{-1}$ EGTA: $2x10^6 M^{-1} s^{-1}$). Therefore, these experiments suggest that the on-rate is more critical in the ability of Ca^{2+} buffers to interfere with the mechanisms underlying Ca^{2+} oscillations. It is possible that within the clusters of IP_3 receptors ("hot-spots") where Ca^{2+} waves are initiated, the close proximity of IP_3 receptors may preclude the action of Ca^{2+} buffers with slow on-rates. A similar correlation between K_{on} and localized Ca^{2+}-buffering has been reported previously by Adler et al., (1991). In this study, BAPTA was found to be more effective at blocking Ca^{2+}-mediated neurotransmitter release in the squid giant synapse than EGTA, and this effect was attributed to its faster Ca^{2+}-binding kinetics.

Modulation of $[Ca^{2+}]_i$ Oscillations by CaBP

Stable expression of CaBP did not appear to have an effect upon the frequency or general pattern of histamine-induced $[Ca^{2+}]_i$ oscillations in HeLa cells. Similar to the case for EGTA, this result is perhaps due to CaBP's binding kinetics, which would not allow it to bind Ca^{2+} rapidly enough between IP_3 receptors in initiation sites to affect initiation of Ca^{2+} oscillations. Once again, by analogy with the study from Adler et al (1991), there is no evidence that the presence of CaBP in neuronal terminals interferes with neurotransmitter release, suggesting that it is likely to act as a relatively slow Ca^{2+}-buffer and is therefore unable to influence Ca^{2+}-dependent events in which the source of Ca^{2+} entry or release is very close to the subsequent target of the action of Ca^{2+}.

Although we were unable to show an effect of CaBP upon initiation of Ca^{2+} oscillations, an effect of CaBP was observed upon global changes in $[Ca^{2+}]_i$ when individual Ca^{2+} spikes were examined. In HeLa-CaBP cells the rate of increase in $[Ca^{2+}]_i$ was slower and the duration of the spikes was prolonged when compared to those observed in the absence of CaBP. Given that the pCINeo-transfected HeLa cells are an appropriate control, we can conclude that any observed differences can be unequivocally ascribed to the effects of CaBP.

In order to quantify the increase in $[Ca^{2+}]_i$ associated with histamine induced Ca^{2+} release, additional experiments were performed utilizing fura-2. These experiments were limited by the effect of the indicator dye on oscillatory patterns, but nonetheless revealed that the peak Ca^{2+} response was significantly lower in HeLa-CaBP cells when

compared to HeLa-pCINeo cells. Overall the effect of CaBP on HeLa cell $[Ca^{2+}]_i$ oscillation is similar to the effect observed in HEK 293 cells; a dampening of the $[Ca^{2+}]_i$ response with transients that are lower in magnitude but longer in duration.

It is possible that the apparent effect of CaBP might be mediated through a direct interaction with the IP_3R, similar to that reported for fluo-3. However, this seems unlikely in light of the similarities of the observed effect upon individual Ca^{2+} oscillations to those $[Ca^{2+}]_i$ transients described in the previous chapter, mediated by caged Ca^{2+} or structurally unrelated channels. CaBP in these experiments had an apparent buffering effect even when the source of the Ca^{2+} was not the IP_3R. Therefore unlike BAPTA, CaBP does not appear to be able to modulate the pattern of Ca^{2+} oscillations owing to its slower binding kinetics, however CaBP clearly modulates the histamine response in HeLa cells by globally buffering Ca^{2+}.

Calcium Waves

It has been previously reported that individual Ca^{2+} oscillations are comprised of Ca^{2+} waves which propagate throughout the cell (Bootman and Berridge, 1996). Activation of phospholipase C following application of histamine results in the production of intracellular IP_3 which, in turn, stimulates the release of Ca^{2+} from the ER. Any Ca^{2+} released from one IP_3R can act synergistically with IP_3 on adjacent receptors, resulting in propagation of the Ca^{2+} wave (Bootman, 1996). Typically these waves have been examined employing confocal laser-scanning imaging, and it has been determined

that the propagation velocity is in the order of 10 μm s^{-1} (Bootman et al., 1997). We have developed a method for resolving Ca^{2+} waves employing a standard epiflourescence Ca^{2+} imaging apparatus in order to determine the effect of CaBP upon wave propagation velocity.

Histamine-induced Ca^{2+} waves were found to be similar to those observed with confocal microscopes in that they had a defined origin, propagated throughout the cell, originated from central or peripheral sites, and were initiated occasionally at more than one site. However, compared to previously reported values the actual velocity of wave propagation was in the order of 80 μm s^{-1}. This is ~8 times faster than the average velocity found by Bootman et al (1997). However the authors of this paper describe the measured wave velocity as a result of a "partially desensitized response": the wave velocity was measured in a cell after having been repeatedly treated with histamine. In our experiments with individual HeLa cells, we also observed that wave velocity decreased with repeated applications of histamine (data not shown).

Mechanism of the Attenuation of Ca^{2+} Wave Velocity

We have shown that EGTA, BAPTA and CaBP all significantly retarded the propagation of Ca^{2+} waves in HeLa cells stimulated with histamine. Propagation of IP_3-mediated Ca^{2+}-waves is reliant upon diffusion of Ca^{2+} from the activated IP_3R to adjacent receptors. The Ca^{2+} -buffering action of EGTA, BAPTA and CaBP appears to inhibit this process, likely by competing with adjacent receptors for Ca^{2+}, inhibiting the

synergistic activation with IP_3. Simple competition for Ca^{2+} however does not take into account the fact that these buffers are mobile in the cytoplasm. Feher (1983) demonstrated using a 3-chamber diffusion model that CaBP can "facilitate" the diffusion of Ca^{2+}. In the model of Ca^{2+} wave propagation, facilitated diffusion would effectively lower the *local* Ca^{2+} concentration surrounding the activated IP_3R limiting the contribution of released Ca^{2+} to synergistic activation of adjacent receptors. Quantitative modeling of Ca^{2+} influx through Ca^{2+}-channels has suggested that the presence of a mobile Ca^{2+} buffer will limit the effect of Ca^{2+} on adjacent receptors (Simon and Llinas, 1985; Roberts, 1994). Therefore the term "facilitated diffusion" is somewhat of a misnomer. Diffusion of Ca^{2+} *is* facilitated by mobile Ca^{2+} buffers, in that such buffers will facilitate distribution of Ca^{2+} throughout the cytoplasm. However, the coefficient of diffusion for CaBP bound to Ca^{2+} ($D=20\mu m^2 s^{-1}$) is an order of magnitude slower than the coefficient of diffusion for free Ca^{2+} in cytoplasm ($D=200\mu m^2 s^{-1}$) (Roberts, 1994). Therefore Roberts (1994) suggests the term "buffered diffusion" as an alternative. The net result of this buffered diffusion in the immediate vicinity of an open Ca^{2+} channel is a reduction in the size of the plume of free Ca^{2+} emanating from the channel and hence a limiting of the effect of this released Ca^{2+} on adjacent receptors. Evidence supporting such an effect of CaBP has been reported in theoretical papers (Roberts, 1994) and experimentally in neurons using electrophysiological techniques (Chard et al, 1995).

As with the effect of buffers upon histamine-evoked Ca^{2+}-oscillations, it appears that the k_{on} value is critical in determining the degree of modulation; the faster on-rate buffer BAPTA has a much greater effect upon Ca^{2+} wave velocity than the slower on-

rate buffers EGTA or CaBP. The k_{on} for CaBP has been estimated at $0.77x10^8$ M^{-1} s^{-1} (I. Mody, personal communication) which is intermediate between BAPTA ($6.0x10^8$ M^{-1} s^{-1}) and EGTA ($2x10^6$ M^{-1} s^{-1}) (Roberts , 1993). This correlation of k_{on} to the efficacy of modulatory effect on Ca^{2+} dependent processes has also been reported in posttetanic potentiation in neurons (Chard et al., 1995).

In the present study, the absolute intracellular concentrations of EGTA or BAPTA are unknown, and would likely have an effect on wave propagation as illustrated by the effects of the two loading concentrations for BAPTA-AM upon Ca^{2+} wave velocity. However, the concentration of CaBP in HeLa-CaBP is similar to that in a number of neurons (see Chapter 2 and Fig. 5) although less than that in cerebellar Purkinje cells. It is likely that increasing the concentration of CaBP would have a similar effect as increasing concentrations of EGTA or BAPTA.

Summary / Conclusions

In summary, it has been demonstrated that CaBP has a significant effect upon the velocity of IP_3 mediated Ca^{2+} waves in HeLa cells, presumably through its ability to buffer Ca^{2+}. In support of this, similar effects were observed in cells loaded with artificial Ca^{2+} buffers, although direct inhibitory effects upon IP_3R (in the case of BAPTA) may have contributed.

The propagation of Ca^{2+}-waves requires the diffusion of Ca^{2+} away from the cluster of IP_3 receptors in the initiation site to other IP_3 receptors that are likely

separated by larger distances. As suggested by others (Adler et al., 1991; Chard et al., 1995), the scope of the effects of Ca^{2+} buffers such as CaBP may be dictated by their binding kinetics. In this case the slower on-rate (relative to fast Ca^{2+}-buffers such as BAPTA) is sufficient to bind and slow down the rate of diffusion of Ca^{2+} to IP_3 receptors involved in the propagation of a Ca^{2+}-wave. In a similar manner, the relatively slow buffering of Ca^{2+} would account for the effect of CaBP on the global Ca^{2+}-responses, namely the reduction in each of the rate of rise, peak value and rate of fall of the individual peaks of an oscillatory Ca^{2+}-response.

The fact that BAPTA was much more effective than CaBP at altering the pattern of an oscillatory Ca^{2+} response, even to the degree of completely preventing them, is consistent with this being an effect largely restricted to the site(s) of initiation. The close proximity of the IP_3 receptors at the initiation sites dictates that Ca^{2+} released from one IP_3 receptor need diffuse only a very small distance to promote the further release of Ca^{2+} from adjacent IP_3 receptors. As such, only a Ca^{2+}-buffer with a sufficient capacity and fast on-rate would be capable of influencing this essential interaction. This effect is entirely analogous to the ability of BAPTA (but not EGTA) to inhibit neurotransmitter release in the squid giant synapse where the calcium channels are spatially located very close to the release sites for the vesicles containing the neurotransmitter (Adler et al., 1991). It is possible that cell-to-cell heterogeneity, along with changing oscillation frequencies over time may have masked any subtle effect of CaBP on oscillatory frequency, and that higher levels of CaBP may be more effective. However, it is more likely that the levels of CaBP naturally expressed in cells, including neurons, are insufficient to influence very rapid Ca^{2+}-dependent effects such as neurotransmitter

release or the initiation of Ca^{2+}-waves. However, given sufficient time, CaBP can be an effective Ca^{2+}-buffer capable of limiting the global Ca^{2+}-response of a cell and of influencing events that require significant diffusion of Ca^{2+} from the source of Ca^{2+} entry or release. Examples would be the ability of CaBP to slow down the velocity of a Ca^{2+}-wave (as described above), and to limit mitochondrial Ca^{2+}-uptake or the activation of other processes leading to Ca^{2+}-induced cell death.

We therefore propose that CaBP may not only be a buffer which protects against possibly harmful increases in $[Ca^{2+}]_i$ but may also affect signal transduction events by modulating Ca^{2+} transients. This has wide ranging significance in all cell types that exhibit Ca^{2+} waves and oscillations (for a review see Berridge et al., 1998). An example in neurons, reported by Gu and Spitzer (1995), demonstrated that the frequency of Ca^{2+} waves can regulate neurite extension and differentiation in spinal neurons. Modulation of this process by CaBP has not as yet been examined, but it is conceivable that modulation of Ca^{2+}-oscillations could influence neurite outgrowth or the expression of developmental genes that do not manifest as structural changes. From this point of view it is of interest that it has been reported that some neurons only transiently express CaBP during early stages of development (Friauf, 1993).

Chapter 5

General Discussion & Conclusions

CaBP and Ca^{2+} Transport

Calbindin-D_{28K} (CaBP) was isolated from chick intestinal epithelium in 1966, where its expression is dependent upon the active metabolite of vitamin D, 1,25-dihydroxy-cholecalciferol (Wasserman and Taylor, 1966). It was subsequently found to be expressed in many vertebrate tissues, including the nervous system, where its distribution has been mapped extensively (Wasserman and Fullmer, 1982; Jande et al., 1981; Celio, 1990). The putative function of CaBP in the intestinal epithelium was hypothesized to be a dual role related to transcellular Ca^{2+} transport; facilitating diffusion of Ca^{2+} across the cytoplasm, while buffering Ca^{2+} and thus preventing large increases in $[Ca^{2+}]_i$ in the transporting cells. In mammals, CaBP is retained in the nervous system but is replaced by a smaller, genetically unrelated, protein calbindin-D_{9K}, in Ca^{2+} transporting cells including those in the gut, kidney and bone (Christakos et al., 1989).

CaBP as a Trigger Protein

Although CaBP was discovered in intestinal tissues, phylogenetic evidence suggests that its expression most likely evolved first in the nervous system (Parmentier, 1990). In the mammalian nervous system, where expression of CaBP is not dependent upon vitamin D, the function of CaBP remains controversial. Evidence from structural

and molecular studies reveal that CaBP is highly conserved across species, demonstrating a 98% sequence identity among mammals and 79% identity between mammalian and avian forms of the protein. Interestingly, this strong sequence conservation is evident in regions of the protein not involved in binding Ca^{2+} (non-EF-hand regions). This observation led Parmentier (1990) to suggest that, in addition to buffering Ca^{2+}, the function of CaBP may involve a protein-protein interaction. This has been supported by Berrgard et al (2000), who utilized spectroscopic analysis of CaBP to examine structural changes following addition of Ca^{2+} (also see Fig. 4, Chapter 2). They found that upon binding Ca^{2+}, or in response to an increase in H^{+}, CaBP undergoes a conformational change which exposes hydrophobic regions of the protein. These conformational changes are similar to, though of a lesser magnitude than those exhibited by "trigger" proteins such as calmodulin. Interestingly, in the absence of Ca^{2+}, calmodulin has very little by way of exposed hydrophobic surfaces, whereas CaBP does. Based therefore on the ability of Ca^{2+}-binding to result in the exposure of hydrophobic surfaces, CaBP falls neither into the classical trigger sub-group of Ca^{2+}-binding proteins, nor into the classical buffer sub-group. More recently, it has been suggested that the presence of hydrophobic surfaces even in the absence of Ca^{2+}-binding, may account for the Ca^{2+}-independent binding of CaBP to caspase-3 reported by Bellido et al (2000). They reported that transfection of osteoblasts with CaBP decreases tumor necrosis factor-α induced apoptosis by inhibiting caspase-3 activity.

CaBP as a Ca^{2+} Buffer

A large body of circumstantial evidence supports a Ca^{2+}-buffering role independent of any cellular effects mediated through possible CaBP-protein interaction. The objective of the present study was therefore to examine the Ca^{2+}-buffering properties of CaBP in cellular systems and to determine the effects of this Ca^{2+}-buffering upon cell survival and Ca^{2+} signaling. Our experiments, taken together, suggest that CaBP can act as a Ca^{2+} buffer and that this Ca^{2+}-buffering property alone may have direct effects on Ca^{2+}-mediated events, both physiological and pathophysiological.

Studies which have compared whole cell $[Ca^{2+}]_i$ with fluorescent probes, to the net flux of Ca^{2+} through channels measured as whole cell Ca^{2+}-currents, have revealed that Ca^{2+}-influx is very effectively buffered by endogenous Ca^{2+} buffers present in all cells. In gonadotrophs, for example, it was found that only 1% of the total number of Ca^{2+} ions which enter a cell remained as the free ion (Tse et al., 1994). Similar studies employing melanotrophs (Thomas et al., 1990), GH3 cells (Lledo et al., 1992) and neurons (Fleet et al., 1998; Fierro and Llano, 1996; Palecek et al., 1999) also revealed this large proportion of Ca^{2+} bound to endogenous buffers. The identity of these endogenous Ca^{2+} buffers is unknown, however studies have shown that they must be immobile and have a low affinity for Ca^{2+} (Kd ~ 100μM in chromaffin cells) since they are not saturated at 1μM $[Ca^{2+}]_i$ (Xu et al., 1997; Neher and Augustine, 1992; Zhou and Neher, 1993). Candidates would include any immobilized, negatively charged cytosolic proteins, perhaps anchored to, or components of, the cytoskeleton.

In light of the studies described above, whatever Ca^{2+}-buffering role CaBP may play, the pool of Ca^{2+} that CaBP is capable of influencing is a very small percentage of the total net Ca^{2+} flux into the cell. Nonetheless, Lledo et al (1992), using CaBP-transfected GH_3 cells, demonstrated that indeed CaBP does have an additional and significant Ca^{2+} buffering effect on this remaining 1% of Ca^{2+}. In our studies, utilizing stable cell lines expressing CaBP, we have unequivocally demonstrated that CaBP can buffer this pool of Ca^{2+}. This buffering was evident in all experiments, manifested by a reduction in rate of $[Ca^{2+}]_i$ rise, a reduction in peak $[Ca^{2+}]_i$ and/or a prolongation of the recovery to baseline $[Ca^{2+}]_i$. Moreover, utilizing the various methods of inducing Ca^{2+} transients, we have provided evidence that this buffering effect is independent of the source of the Ca^{2+} entry or release. Furthermore, our experiments demonstrate this source-independent Ca^{2+} buffering can have consequences upon physiological (Ca^{2+} waves and oscillations) and pathophysiological (NMDA mediated excitotoxicity) events.

From a theoretical point of view, a Ca^{2+}-buffer would be expected to act to lower peak $[Ca^{2+}]_i$ and reduce the rate of rise and decay of $[Ca^{2+}]_i$. In a variety of experimental models, we have demonstrated that transfected CaBP can produce some or all of these effects. Most notably, we observed that in HEK treated with ATP or 4-Br-A23187, transfected CaBP produced all of the effects ascribed to a Ca^{2+} buffer. Therefore, due to the use of simple cellular systems in which the only variable altered between control and experimental groups was the presence or absence of CaBP, this study provides unequivocal evidence in favour of a Ca^{2+}-buffering role for CaBP.

Additionally, our studies demonstrating Ca^{2+} wave attenuation provide evidence that CaBP is acting as a mobile Ca^{2+} buffer. As described above, the vast majority of

Ca^{2+} entering the cell is bound by immobile endogenous buffers. A fixed buffer would retain a high concentration of Ca^{2+} locally while a mobile Ca^{2+} buffer would be expected to have the effect of facilitating "buffered diffusion" of Ca^{2+} (Roberts, 1993). This would limit the local rise of Ca^{2+} around activated IP_3 receptors and therefore the activation of adjacent receptors (Roberts, 1993; Hall, et al 1997; Wang and Thompson, 1995) and attenuate the rate of Ca^{2+} wave propagation.

Possible Consequences of Ca^{2+} Buffering by CaBP

Intracellular Ca^{2+} is a signaling molecule for a wide variety of biological processes. However, it has been shown that a prolonged elevation of $[Ca^{2+}]_i$ can be harmful to cells and Ca^{2+}-induced cell death has been implicated in numerous pathologies (reviewed by Doble, 1999). Many studies have also examined the potential correlation between the expression of CaBP and cell survival in many neuropathologies (reviewed by Heizmann and Braun, 1992). There is evidence of neuronal sparing of cells expressing CaBP in Parkinson's disease (Yamada et al., 1990; German et al., 1992), and MPTP-induced Parkinson's (German et al.,1992; Iacopino et al., 1992; Lavoie and Parent, 1991), while others have shown altered expression of CaBP in Alzheimers disease (Lally et al., 1997; Ichimiya et al., 1988), epilepsy (Sloviter et al., 1991; Mody et al., 1987; Magloczky et al., 1997), and Down syndrome (Kobayashi et al., 1990). However, there are many inconsistencies in the argument for a neuroprotective role for

CaBP based upon anatomical data, likely due to the correlative nature of many of these studies.

In addition to these correlative observations between CaBP and neuropathologies, a number of experimental approaches have been made in order to determine whether or not either artificial Ca^{2+} buffers, or naturally occurring molecules such as CaBP that may act as Ca^{2+} buffers, are capable of preventing Ca^{2+} mediated cell death. It has been hypothesized that CaBP may mediate this resistance through its ability to buffer increases in intracellular calcium (Baimbridge and Parkes, 1981; Baimbridge et al., 1982; Jande et al., 1981; Mody et al., 1987). Following this rationale, several studies have examined the effect of artificial calcium buffers in preventing Ca^{2+}-mediated cell death. The potential utility of the rapid intracellular calcium buffer BAPTA as a (neuro)protective agent remains controversial, as recent studies have reported protective (Tymianski et al., 1993b) or deleterious (Adbel-Hamid and Baimbridge, 1997) effects of BAPTA in the face of Ca^{2+} challenges. Similar contradictory reports of the protective properties of *endogenous* CaBP have been published using various cell models (Mattson et al., 1991; Möckel and Fisher, 1994; Abdel-Hamid et al., In Preparation). However, recent studies, including those presented in Chapter 3, employing molecular biological techniques to introduce CaBP into cells which do not normally express CaBP, have provided evidence that transfected CaBP enhances survival following influx of toxic levels of Ca^{2+} (McMahon et al., 1998; Meier et al., 1998).

In our studies, it would appear that CaBP is protective in our transfected HEK 293 model of Ca^{2+}-mediated cell death. Specifically, CaBP appears to prevent *delayed*, presumably apoptotic cell death cell death 24 hours following excitotoxicity treatments.

These experiments are in agreement with the results reported by Gwag et al (1999), in that there is a Ca^{2+}-toxicity threshold, above which cells die by necrosis, whereas more moderate $[Ca^{2+}]_i$ challenges induce apoptotic cell death. In our experiments, CaBP was unable to prevent cell death which occurred within 6 hours following excitotoxic treatments, presumably due to entry of Ca^{2+} in excess of the total buffering capacity of CaBP. However, in cells that were exposed to more moderate levels of $[Ca^{2+}]_i$, our study suggests that CaBP was able to successfully buffer levels of $[Ca^{2+}]_i$ and prevent cell death that is likely to be apoptotic in nature.

CaBP, Mitochondria and Cell Death

In the past decade it has been established that mitochondria, in addition to their essential role as generators of ATP, are also capable of sequestering and therefore contributing to the buffering of Ca^{2+} transients (Thayer and Miller, 1990, Werth and Thayer, 1994; Sidky and Baimbridge, 1997). However, it has also been reported that mitochondria constitute a potential "cellular poison cupboard", which, in response to physiological or pathophysiological signals (including Ca^{2+}-uptake) will induce apoptotic or necrotic cell death (Earnshaw, 1999; Miller, 1998; Nicholls and Bud, 1998; 2000). In support of this function, it has been recently demonstrated that inhibition of mitochondrial Ca^{2+} uptake can prevent excitotoxic cell death (Stout et al., 1998). This mechanism raises the possibility that CaBP may act to prevent cell death by inhibiting uptake of Ca^{2+} into mitochondria. This hypothesis was first proposed by Rasmussin and

Gustin (1978), who, by comparing the relative Ca^{2+}-affinities of CaBP (Kd ~0.5 μM) and mitochondria (Kd ~1.0 μM), suggested that the preferential binding of Ca^{2+} to CaBP may prevent Ca^{2+}-uptake into mitochondria in Ca^{2+}-transporting cells. This is one of the potential mechanisms to explain the results of the Ca^{2+} excitotoxicity studies described in Chapter 3, where CaBP appeared to prevent delayed cell death. By preventing Ca^{2+} uptake into mitochondria, CaBP may serve to protect the cell against death if Ca^{2+} uptake into mitochondria is absolutely required for death to occur, as has been shown by Stout et al (1998).

CaBP as Modulator of Intracellular Signaling

Another potential role for CaBP suggested by the studies presented in this thesis is that of a modulator of intracellular Ca^{2+} signals. In transfected HeLa cells, CaBP significantly attenuated the velocity of histamine-induced Ca^{2+} waves. As well, we observed a Ca^{2+}-buffering effect upon Ca^{2+} oscillations, with peak $[Ca^{2+}]_i$ and the rates of increase and recovery of $[Ca^{2+}]_i$ all being reduced. We did not observe a direct effect upon the frequency of Ca^{2+} oscillations, likely due to the binding kinetics of CaBP which would not allow it to influence rapid Ca^{2+} events between the closely spaced IP_3R at initiation sites. However this is consistent with studies that have suggested that buffers with slow on-rates may not be able to influence local, rapid Ca^{2+}-mediated events but can effect global changes or events that involve greater distances between the source of Ca^{2+} entry and the effector molecules (Adler et al., 1991; Chard et al., 1995).

As discussed in Chapter 4, CaBP may, in addition, be capable of modulating the frequency of Ca^{2+} oscillations, given a high enough intracellular concentration of the protein. CaBP-mediated modulation of Ca^{2+} oscillations is of particular interest in the light of recent studies that have demonstrated variations in amplitude (Dolmetch et al., 1997) and/or frequency (Dolmetch et al., 1998, Li et al., 1998) of calcium oscillations can modulate gene expression. Another report by Gu and Spitzer (1995), demonstrated that modulation of calcium oscillations can affect neurite outgrowth. Taken together with our findings, this raises the possibility CaBP could affect gene expression and hence development through modulation of calcium transients or oscillations. Initial investigation of CaBP knockout mice have not revealed any structural abnormalities (Klapstein et al., 1998), however the possibility remains that CaBP could influence gene expression during development which is not manifested by structural changes. An interesting example of a specific temporal and spatial pattern of CaBP expression during development is the transient appearance of CaBP in the superior olivary complex of developing rats (Friauf,1993). This expression could also be interpreted as a safeguard against cytotoxic levels of Ca^{2+} during active Ca^{2+}-signalling throughout development.

Future Studies

Studies in Transfected HEK Cells

Further studies on HEK 293 stable cell lines should focus upon the exact mechanism of cell death. The type of cell death (necrotic/apoptotic) in response to activation of transfected NR1/NR2A receptors should be examined. The influence of CaBP upon mitochondrial activity in transfected HEK 293 cells could be examined in two ways. Firstly, mitochondrial Ca^{2+} could be examined directly using the Ca^{2+}-indicator dye Rhod-2 that, in its reduced form, is preferentially taken up by the mitochondria. If CaBP does indeed delay mitochondrial Ca^{2+} uptake, then we would expect that mitochondrial Ca^{2+} would not increase in parallel with increases in cytoplasmic Ca^{2+}, measured with cytoplasmic-specific dyes. Secondly, the Ca^{2+}-mediated disruption of the mitochondrial membrane potential that is hypothesized to be a step in apoptotic and necrotic cell death should be examined. Rhod-123 is a mitochondrial membrane potential sensitive dye which is significantly quenched inside mitochondria. Upon disruption of the inner membrane potential, the dye is released into the cytoplasm where its fluorescence intensity increases. If CaBP is capable of binding Ca^{2+} and preventing mitochondrial Ca^{2+} uptake, we would expect that the transfection of CaBP would prevent or delay the depolarization of the mitochondrial membrane potential, thereby preventing or delaying cell death.

Excitotoxicity studies of transfected HEK cells to date have employed simple cell counts to assess cell death. These studies should be followed up with experiments utilizing the live cell indicator 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT), that is metabolized to a dark blue formazan product by live cells which can be quantified by absorption spectroscopy. Furthermore, NR1/NR2A transfected HEK cultures subjected to excitotoxic stimuli should be examined for specific signs of apoptotic cell death. This could be carried out by staining with 4,6-diamindino-2-phenylindole (DAPI) to determine the degree of peripheral nuclear condensation and Western Blotting for cytochrome C released from the mitochondria. Another possibility is the examination of caspase-3 activation, which is also associated with apoptotic cell death. This might be particularly interesting in light of the recent observations of Bellido et al (2000) that CaBP may exert its anti-apoptotic action through inhibition of caspase-3 activation.

Studies in Primary Hippocampal Cultures

Concurrent with the studies described in this report, we have recently generated a replication deficient adenovirus that will be employed to express CaBP in primary hippocampal cultures. Additional viruses are being generated which will co-express green fluorescent protein (GFP) and CaBP. This will allow for assessment of exogenous CaBP expression in live cells. The efficacy of CaBP-expressing herpes simplex viruses in preventing glutamate-mediated cell death have been recently described (Meier et al.,

1998). These finding should be confirmed and extended to studies of the effect of CaBP upon mitochondria function, employing the mitochondrial-specific dyes described above.

In Vivo Studies

The hypothesized protective role of CaBP could also be tested in *in vivo* models of neuronal cell death. Stereotacic injections of CaBP expressing adenovirus could be employed to assess the protective effects of CaBP in ischemia models in rats and in the MPTP mouse model of Parkinsons Disease. These experiment could also be performed with the newly-available CaBP-knockout mice to eliminate any effects of endogenous CaBP.

Conclusions and General Summary

1. To overcome the potential confounding phenotypic effects of cells which naturally do or do not express CaBP, human CaBP was cloned from a cDNA library, inserted into mammalian expression vectors, and used to generate stable cell lines (with appropriate control cell lines). Therefore any differences between control and experimental groups in the experiments described can be unequivocally ascribed to the effect of CaBP.

2. We have obtained evidence that directly supports a Ca^{2+} buffering role for CaBP. This Ca^{2+} buffering was evident in all experiments, despite different routes of Ca^{2+} entry or release.

3. In a model of excitotoxicity employing transfected NMDA receptors, the presence of transfected CaBP has been directly correlated to protection from delayed, presumably apoptotic cell death. This effect is possibly due to prevention of Ca^{2+} uptake by mitochondria.

4. We have explored the possibility that CaBP may influence other modes of Ca^{2+} signaling, particularly through the generation of Ca^{2+} oscillations and Ca^{2+} waves in stable-transfected HeLa cells. While we were unable to measure any direct effect upon the frequency of Ca^{2+} oscillations, the profile of the component Ca^{2+} spikes was flattened and CaBP significantly attenuated the velocity of Ca^{2+} waves.

5. Our results are consistent with a Ca^{2+}-buffering action of CaBP that, compared to BAPTA, is relatively slow. Thus, events that are spatially coupled to the source of Ca^{2+} entry or release are influenced by BAPTA but not CaBP. Examples would be neurotransmitter release and the feedback effects of Ca^{2+} on IP_3 receptors.

6. When there is significant diffusion of Ca^{2+} between the source of entry or release and the target, (e.g. mitochondria or IP_3 receptors activated during an intracellular Ca^{2+} wave) CaBP may have a significant influence. An example would be the ability of CaBP to reduce the velocity of a Ca^{2+} wave.

One of the intriguing facets in the study of intracellular signaling is the fact that a single ubiquitous messenger such as calcium mediate a myriad of diverse cellular processes, including cell death. Four possible mechanisms have been proposed : amplitude modulation, frequency modulation, control of route of calcium entry (or release) and differential expression of downstream signaling molecules (reviewed by Berridge, 1997). In this study it has been demonstrated that CaBP can influence Ca^{2+} mediated cellular processes through the spatial and temporal buffering of cytosolic Ca^{2+}. The fourth possibility (not addressed experimentally in this work) may explain the differential susceptibility of certain cell types to cytotoxic calcium. Absence of expression of downstream signaling molecules in the cell death pathway may effectively protect cells regardless of CaBP expression. Similarly, the ability of CaBP to modulate Ca^{2+} signals may depend upon the expression levels and sensitivity of the target

mechanisms. In summary, the *context* of expression of CaBP may prove to be a very important determinant in the protective or modulatory capabilities of CaBP.

Abbreviations

AM	Acetoxymethyl ester
APV	2-Amino-5-phosphonovalerate
ATP	Adenosine triphosphate
BAPTA	1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BBSS	Balanced Buffered Salt Solution
BSS	Buffered Salt Solution
Ca^{2+}	Ionized calcium
$[Ca^{2+}]_i$	Intracellular ionized calcium concentration
CABP	Calbindin-D28K
cDNA	Complementary Deoxyribonucleic Acid
CRAC	Calcium Release Activated Channel
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl suifoxide
DNA	Deoxyribonueleic acid
dNTP	Deoxynucleoside triphosphate
EAA	Excitatory amino acids
EGTA	Ethyleneglycol-tetraacetic acid
ER	Endoplasmic Reticulum
FBS:	Fetal bovine serum
HBS	Hepes Buffered Saline
HEK 293	Human Embryonic Kidney (cells)
IP_3	Inositol 1,4,5-triphosphate
IP_3R	Inositol 1,4,5-triphosphate-Receptor
Kd	Dissociation constant
LTP	Long Term Potentiation
Mito	Mitochondrion
mRNA	Messenger ribonucleic Acid
NMDA	N-Methyl D-Aspartate
NMDAR	N-Methyl D-Aspartate Receptor
NP-EGTA	*o*-nitrophenyl EGTA
PCR	Polymerase Chain Reaction
PBS	Phosphate Buffered saline
PM	Plasma Membrane
PMCA	Plasma Membrane Ca^{2+}-ATPase
PTP	Mitochondrial Permeability Transition Pore
ROCC	Receptor-operated Ca^{2+} channel
ROI:	Region of interest
RyR	Ryanodine Receptor
SEM:	Standard error of the mean
SERCA	Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase
VOCC	Voltage-operated Ca^{2+} channel

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