CALCIUM SIGNALLING AND BUFFERING:

THE ROLE OF ANNEXIN VI

by

Amanda Fleet

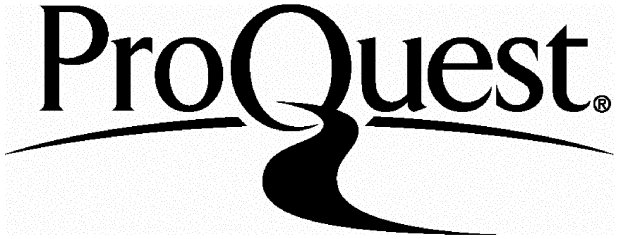
A thesis submitted in partial fulfilment

of the requirements for the degree of

Doctor of Philosophy

University College, London

1997



ABSTRACT

Intracellular free calcium concentrations and their changes are important since calcium is a ubiquitous second messenger in eukaryotic cells. Raised intracellular calcium concentrations have been implicated in cell division, gene regulation, exocytosis and many other cell functions. The magnitude of the free calcium concentration change observed after stimulation depends on two things: the amount of calcium influx into the cytoplasm, and the extent to which this calcium is buffered by cell constituents.

Calcium influx into the cytoplasm occurs in two ways: release of stored calcium from intracellular organelles such as the endoplasmic reticulum, and calcium entry into the cell across the cell membrane from the extracellular environment. A431 cells, a human epithelial cell line, respond to epidermal growth factor (EGF) with two phases, an initial rise in intracellular free calcium concentration which is followed by a maintained plateau of raised intracellular free calcium. The initial, peak calcium response is due to calcium release from internal stores, whereas the maintained calcium change is from calcium entry into the cell across the plasmalemma.

A431 cells transfected with the protein annexin VI, a member of a family of proteins that bind to phospholipids and calcium, grow more slowly than wild type A431 (wtA431) cells and have a reduced calcium response to EGF. The hypothesis that annexin VI was acting as a calcium buffer was tested and it was found that there was no difference in calcium buffering capacity between wild type and transfected (annexin VI^+) cells. Annexin VI^+ cells have a pattern of response to EGF that is distinct from the wild type cells in displaying no sustained

calcium elevation following stimulation. The hypothesis suggested from the results of these experiments is that calcium influx across the cell membrane is inhibited by annexin VI, thereby removing the maintained calcium increase within the cells following stimulation. The prolonged raised calcium might be necessary for cell division and cell regulation.

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LIST OF ABBREVIATIONS

$[Ca^{2+}]i$	intracellular free calcium concentration
AM	acetoxy-methyl
ATP	adenosine triphosphate
BAPTA	1,2-*bis*(*o*-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BK	bradykinin
BK-R	bradykinin receptor
CICR	calcium-induced calcium release
DAG	diacylglycerol
EGF	epidermal growth factor
EGF-R	epidermal growth factor receptor
EGTA	ethylene glyceroltetraacetic acid
F	Fluo-3
FCS	foetal calf serum
HBS	HEPES buffered saline
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
$InsP_3$	inositol (1,4,5) trisphosphate
$InsP_3$-R	inositol (1,4,5) trisphosphate receptor
LC_4	leukotriene C_4
NP-EGTA	nitro-phenyl EGTA
OD	optical density
PABA	*p*-aminobenzoic acid
PGE_2	prostaglandin E_2

PIP_2	phosphatidyl inositol 4,5-bisphosphate
PITP	phosphatidyl inositol transfer protein
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PMT	photomultiplier tube
rhEGF	recombinant human epidermal growth factor
SR	sulforhodamine 101
TG	thapsigargin
TPA	phorbol-12-myristate-13-acetate
TRIS	*tris*(hydroxymethyl)aminomethane
UV	ultraviolet

INTRODUCTION

Calcium is an important second messenger in cells. Since it is the second messenger employed by a multitude of signal transduction pathways, it is crucial that cells maintain and control particular levels of the ion depending on circumstances. Since it is the second messenger in so many signalling pathways, obviously the magnitude and pattern of calcium changes in a cell are important, enabling the cell to distinguish between different messages. Hence, anything that affects the magnitude, duration or pattern of a calcium response within a cell could have profound effects downstream.

This thesis examined several aspects of calcium signalling, within three basic projects. In the project first I measured the calcium buffering capacity of a neuronal cell line. In the second I examined the relationship between receptors for two agonists, bradykinin and epidermal growth factor (EGF) on A431 cells. In the third I investigated the effect of expression of the protein annexin VI on A431 cells. These projects are linked loosely because all are connected with calcium signalling. They are also linked more tightly in that I used the techniques I developed in the study of buffering to address the question of whether annexin VI was a significant calcium buffer in transfected A431 cells, while the other two studies address the interactions of annexin VI, the EGF receptor and the bradykinin receptor in initiating calcium signals in A431 cells.

It is essential that cells manage to buffer a calcium load, since high levels of calcium in cells are cytotoxic. The higher the buffering capacity of a cell for calcium, the more likely the cell is to survive a large calcium load. Conversely, a

low ability to buffer intracellular calcium loads would be associated with a greater incidence of cell mortality.

The total calcium load upon a cell depends on two factors - the length of time over which the calcium load is applied and the size of the load. The remainder of this thesis examined conditions which would affect these two factors - interactions between receptors/signalling pathways, and the effect of expression of a protein implicated in growth modulation.

Interaction of a growth factor with a receptor and signalling pathway principally activated by a different agonist, may affect both the magnitude and duration of the response to this second agonist when subsequently presented. This was investigated in A431 cells for the two growth factors bradykinin and epidermal growth factor.

The expression or absence of expression of modulatory proteins in a cell will obviously have profound effects on the signalling pathways within the cell. Over-expression of an inhibitory protein will lead to reduced growth and division whereas absence of such a protein will lead to increased growth and division. The effect of expression of a protein implicated in the modulation of growth was examined, with respect to changes to the calcium signalling responses following application of growth factors, to characterise the modulation observed.

It is evident that the subtle interplay of all the factors outlined above have significant consequences for the growth, division and survival of cells and it was the aim of this thesis to examine some of this interplay to understand the consequences.

1.1 CALCIUM BUFFERING

1.1.1 Calcium buffering in cells

Calcium is an important second messenger in cells. Consequently it is essential for cells to be able to buffer and remove calcium from the cytosol, to maintain a resting free calcium level ($[Ca^{2+}]_i$) of approximately 100nM.

Most of the calcium in cells is either bound to buffers or stored in discrete, membrane bound organelles, for example mitochondria or endoplasmic reticulum. $[Ca^{2+}]_i$ levels can rise after stimulation of the cell, either by entry of calcium from the extracellular fluid through calcium channels in the cell membrane or by release of calcium from internal stores. The spatial and temporal characteristics of intracellular calcium signals depend critically on the degree to which calcium is buffered in the cytosol. Nevertheless, there have been few measurements of cytosolic calcium buffering. Knowledge of calcium buffering is important to be able to predict the extent to which a calcium wave will progress into the interior of the cell and what the change in free calcium in a cell will be when a known calcium load is applied to the cell. Therefore, one can predict which downstream targets might be activated.

When the calcium concentration in the cytosol rises, calcium ions bind more or less avidly to cellular constituents such as protein and phospholipid, and are thus removed from the free calcium ion pool. I will use the term physicochemical calcium buffering for this process. Ultimately, the excess calcium must be removed from the cytosol. This involves the pumping of calcium ions out of the cytosol into two places: intracellular stores or across the cell membrane into extracellular fluid. Therefore the ability of a cell to deal with a calcium load

depends both on the size of the load and the ability of the cell to deal with the calcium through physicochemical buffering and pumping from the cytosol. The greater the capacity for binding calcium ions until they can be removed from the cytosol through the action of pumps, the more likely the cell is to survive a large calcium load. Conversely, a low buffering capacity in a cell would lead to a greater likelihood of cell damage or death following trauma.

1.1.2 Measurement of intracellular calcium using calcium-sensitive fluorescent dyes

(a) General properties of fluorescent probes:

Much has been learned of the role of calcium as an intracellular messenger, through the use of calcium indicator dyes, such as Fura-2, Indo-1, Fluo-3 and Quin-2. The dyes were created by Tsien and colleagues and most are analogues of the calcium chelator EGTA, with substitution of various groups to give a compound that displays changes in fluorescent properties on binding calcium and a greater specificity for calcium over magnesium and hydrogen ions. The changes in fluorescent properties are generally of two forms. On binding calcium, single wavelength dyes change the intensity with which they fluoresce with no significant change of excitation and emission spectrum. Ratiometric dyes exhibit a change of either excitation spectrum, emission spectrum, or both, upon binding calcium. With a ratiometric dye such as Fura-2 or Indo-1 the ratio of intensity at one wavelength to the intensity at another can be used to determine the concentration of calcium present, regardless of the concentration of the dye. Fura-2 has a wavelength shift in the optimal excitation light - as calcium binds to

the dye, the optimal excitation wavelength shortens. The colour of the light emitted by the dye remains approximately constant. Hence a ratio of fluorescence emission during successive excitation at two different wavelengths can be used to determine intracellular calcium concentrations. The calcium-bound form of the dye has an excitation maximum of 335 nm; the calcium-free form of the dye has a maximum of 362 nm. 380 nm is the wavelength at which the two spectra differ the most and so is a convenient wavelength at which to measure. The iso-fluorescent wavelength of the dye (the calcium insensitive wavelength for excitation) is 360 nm. Since fluor optics transmit <340 nm light poorly, the wavelength pair 350/380 nm is commonly used (in some experiments discussed here, the fluorescence pair is 340/380). Fluorescence emission is recorded at 510 nm. For Indo-1, in contrast, the colour of the emitted light changes when the dye binds calcium. The peak emission of calcium-free dye is at 485 nm and of calcium-bound dye is at 405 nm. The iso-fluorescent point is 445 nm. Since the two emission signals can be acquired simultaneously, Indo-1 has a number of uses in monitoring cellular events which demand a high temporal resolution.

For a number of reasons Fura-2 is used more widely than is Indo-1 and is the dye used in some of the experiments presented here. However, the necessity for UV for exciting these ratiometric dyes makes them unsuitable for a number of applications such as the use of "caged compounds" which utilise UV light to photolyse the compound to release the caged molecule.

The commonly used visible wavelength dyes are single wavelength. This means that there is no wavelength shift of either excitation or emission with the calcium binding, but an increased intensity of emission fluorescence. This has inherent problems since a fall of fluorescence from a cell may mean a fall of

calcium concentration within the cell but may equally indicate a loss of the dye - which can occur due to bleaching or active pumping of dye across the plasma membrane. In some of the experiments reported here, Fluo-3 has been used as the calcium indicator, but to overcome the problems associated with a non-ratiometric dye, it has been used in conjunction with a calcium-insensitive marker dye against which ratios of fluorescence intensity can be made.

(b) Methods of introduction of the dye onto the cell:

Introduction of the free acid into the cytoplasm:

In this project free acid was introduced into the cell by micro-injection. This involves the insertion of a fine glass micropipette into the cell. The micropipette contains dye in the tip so that applying a back pressure to the pipette forces a small quantity of dye into the cell. The advantages of this method are as follows. There is the possibility of being able to calculate the quantity of dye introduced into the cell by co-injecting a marker dye whose fluorescence is not altered by the concentration of ions in the cytoplasm. This can be of particular importance with the single wavelength dyes, whose fluorescence does change according to the presence of intracellular ions. There are also a number of cell types that do not de-esterify the acetoxy-methyl (AM) forms of the dyes used in passive loading, in which case the only option is to introduce dye into the cell directly. Problems of dye compartmentalisation are reduced with this method as compared with AM loaded cells, but are not avoided completely (see section 1.1.2 (c) below). The disadvantages, however, include cell damage; the necessity for large, robust cells

that are firmly attached to a surface to allow for microinjection and the limitations on the quantity of cells that can be injected within a time limit.

Other methods of introduction include diffusion from a patch pipette in whole-cell mode, scrape-loading, ATP loading and permeabilisation of the membrane through voltage changes or detergents. There are few advantages to these methods other than reduced comparmentalisation, and the ability to use this method on many cell types, not just large and robust ones. There are a number of inherent disadvantages though, such as low cell viability and cell damage.

Loading cells with a membrane permeable form:

A membrane permeable form of the dye which has ester groups attached to the parent compound can be used to load a cell with dye through an intact and unmodified plasma membrane. The acetoxy-methyl ester form of the dye is used (Tsien, 1981). This is hydrophobic and so can pass through the plasma membrane. Once inside the cell, it is de-esterified to produce the free acid and formaldehyde. The cell retains the free acid, which is now fluorescent and calcium sensitive. The advantages associated with this method include its convenience - large numbers of cells can be loaded at the same time and many cell types can be used; there is no requirement for specialist equipment and relatively little damage is caused to the cell by loading. The disadvantages with this method include problems with compartmentalisation of the dye, incomplete hydrolysis of the AM form leaving fluorescent but non-calcium sensitive species in the cell and endocytosis of the AM form from the media so that the esterified dye enters the endocytic pathway and is moved into high calcium compartments within the cell. This results in the same effect as compartmentalisation.

(c) Compartmentalisation:

Compartmentalisation occurs when dye moves into organelles within the cell, such as mitochondria or endoplasmic reticulum. Since these organelles have a calcium concentration that is different from that of bulk cytosol, compartmentalisation leads to erroneous indications of both static calcium concentration within the cell and errors in the changes of calcium observed. Many organelles have a high calcium concentration that may saturate the dye and cause the indicated concentration of calcium in the cell to be much greater than the free calcium concentration in the cytosol actually is. Small changes in the cytosolic levels of free calcium may thus be masked. Dye can enter the organelles via two major routes. The free-acid form of the dye can be pumped into the organelles by the cell, or, when ester-loading cells, the esterified dye may cross both the plasma membrane and also the membranes surrounding these organelles. Sulfinpyrazone may be used to inhibit the pumps that are pumping the dye out of the cytosol either into these stores or out of the cell completely.

1.1.3 Previous methods of determination of calcium buffering

(a) Basic approach:

Buffering has been used to mean two different things - "fast" buffering (or physicochemical buffering) where cell constituents bind calcium, and "slower" buffering, where calcium is pumped out of the cytosol either into calcium stores within the cell, such as mitochondria or endoplasmic reticulum, or across the plasma membrane into extracellular fluid. In this thesis I am only concerned with physicochemical buffering.

To be able to determine the buffering of a cell, one has to know two things - the total calcium load added to cytosol, and the change in intracellular free calcium that results. Most previous studies of cytosolic calcium buffering have used voltage gated calcium channels in the plasma membrane to deliver the calcium load. This allows the size of the calcium load to be calculated from the electrical current across the membrane. However this approach generates a spatially inhomogenous rise in calcium across the cell. Measuring calcium buffering capacity by imposing a spatially inhomogenous calcium load raises the possibility of two types of error. First, calcium buffers are unlikely to be distributed homogenously throughout the cell. Calcium buffers may well be associated with microdomains of high calcium in a cell. Rizzuto *et al* (1993) suggest that there are such microdomains created when inositol 1,4,5-trisphosphate ($InsP_3$) gated channels open and that these microdomains are sensed by mitochondria nearby. Tillotson and Gorman (1980) suggest that in nerve cells, systems involved in buffering calcium are not distributed uniformly and that in the soma of a molluscan pacemaker neurone, buffering systems are localised principally near the inner surface of the plasma membrane. This is consistent with Roberts (1993), who found that frog saccular hair cells contain high concentrations of a mobile cytoplasmic calcium buffer close to pre-synaptic calcium channels. The result of any method of measuring calcium buffering capacity that involves applying a spatially inhomogeous calcium load will therefore depend critically on the degree of overlap of the load and the buffering capacity of the cytosol; if overlap is poor, buffering capacity will be underestimated.

The second type of error can arise even if cytosolic buffers are uniformly distributed. Buffers situated at the site of the calcium load, e.g. immediately below the plasma membrane, may be saturated while buffers away from the calcium load, e.g. at the cell centre, are not exposed to the calcium rise. Thus total cell buffering is likely to be underestimated.

(b) Measurements on muscle cells:

Work by Berlin and colleagues (Berlin, Bassani and Bers, 1994) examined the physicochemical calcium buffering of rat cardiac myocytes. They prevented uptake of calcium into internal stores, then depolarised the cell, causing calcium to enter the cytoplasm through voltage operated calcium channels (VOCC). Their method was to patch clamp the cells and measure the current from the entry of calcium, thereby determining the amount of calcium that had entered the cell. They measured Indo-1 fluorescence ratios to calculate the calcium concentration within the cell and compared observed calcium concentration changes with predicted calcium concentration changes according to knowledge of introduced calcium and cell volume. The values they calculated for intrinsic buffering gave the sum of the capacities of all the buffers (Bmax) as 123 ± 18μmol/l cell water with a Kd of 0.96 ± 0.18μM, or, to raise intracellular calcium concentration from 0.1 to 1μM, approximately 50μM calcium would need to be added to the cytosol. However, their method had two problems - the calcium was being driven up at the periphery and has the potential problem of saturation of buffers, and calculating cytosol volume is made troublesome by the convoluted network of t-tubules and

the significant volume of cell that is occupied by the contractile cytoskeleton, mitochondria, and the sarcoplasmic reticulum.

Hove-Madsen and Bers (1993) also examined passive calcium buffering in ventricular myocytes (although from rabbit not rat). Their method examined permeabilised cells and compared the calcium concentrations seen in a cell suspension when calcium was added compared to the calcium concentration seen in a blank containing no cells. The difference between the two conditions is due to components of the cell buffering the added calcium, hence indicating a reduced change in free calcium concentration. With this method, both the removal of free calcium from the cytoplasm by binding to cell constituents and through the action of pumps would be measured, so the uptake into internal stores was blocked by thapsigargin and ruthenium red. However, this still left two "pools" of calcium buffers unaffected - the fast buffers which have calcium specific sites and will bind calcium rapidly (calcium specific binding sites of troponin and calmodulin for example), and slow buffers which have Ca/Mg sites (Ca/Mg binding sites of troponin and myosin for example) and which at low calcium levels will generally have Mg^{2+} bound. Calcium binding to these sites occurs only after the dissociation of Mg^{2+}.

Hove-Madsen and Bers fit the passive calcium binding data they obtained with a Michaelis-Menton binding curve with two sites: $K_1 = 0.42\mu M$, $n_1 = 1.27$ nmol/mg cell protein and $K_2 = 79\mu M$, $n_2 = 4.13$ nmol/mg cell protein. This relates to 97μmol calcium per litre of cell water being needed to be added to raise the calcium concentration within the cell from 0.1μM to 1μM, a value approximately double that of Berlin, Bassani and Bers (1994).

Balke, Egan and Wier (1994) examined calcium binding to endogenous ligands quantitatively, comparing the observed $[Ca^{2+}]_i$ changes for a known total amount of calcium entering the myocytes with the predicted calcium change, assuming calcium could bind to endogenous intracellular calcium ligands and to Indo-1, the calcium sensitive dye used for monitoring the calcium concentration within the cell. They assumed that calcium could bind to troponin C, calmodulin, the sarcoplasmic reticulum and the surface membrane of the cells, and compared what the change in free calcium against time measured was, with the calculated change using rate equations for the binding of calcium to the ligands mentioned. Their results are fitted by endogenous buffering capacity of approximately 145 - i.e. that at an intracellular calcium concentration of 0.2μM, it would need 145 calcium ions to enter the cell, to raise the number of free calcium ions within the cytoplasm by one.

(c) Measurements on neurones:

Ahmed and Connor (1988) measured the calcium buffering capacity of the cytosol of molluscan neurones through an indirect method. They added EGTA to the cytosol to reduce the transient changes in free calcium produced by voltage pulses applied to the cell, then calculated what the buffering capacity of the cell was, assuming a linear relationship between exogenous and endogenous buffer capacities such that they could be summed. The buffering capacity of the cell was calculated for three assumptions: 1) that the calcium entering the cell was distributed uniformly throughout the cytosol; 2) that the calcium entering the cell was distributed within a shell of 5μm thickness from the inner plasma membrane

surface; and 3) that the shell described was of 10μm thickness. The buffering capacity calculated for each of these assumptions were 118.5μM/ΔpCa; 34.5μM/ΔpCa and 45.2μM/ΔpCa respectively.

Stuenkel (1994) examined the buffering capacity of isolated rat neurohypophyseal nerve endings by opening voltage operated calcium channels via step depolarisations of voltage clamped cells and found that at low calcium loads, more than 99% of calcium influx was buffered by an endogenous buffer component - probably intracellular calcium binding proteins. The method of analysis of the patched cells followed that of Neher and Augustine (1992) and indicated that the calcium buffer was relatively or absolutely immobile since it was not lost from the cells during 300 s of whole cell recording. The buffering capacity provided by these buffer(s) was estimated to be 174 - i.e. 174 calcium ions have to enter the cell, to raise the number of free calcium ions in the cytosol by one. At higher calcium loads, a second calcium buffering mechanism appeared which exhibited high capacity, but low affinity for calcium and limited increases in $[Ca^{2+}]_i$ to approximately 600nM. Since it was sensitive to ruthenium red, it is assumed that it is the calcium pump on the mitochondrial membrane.

(d) Philosophy behind the present work:

For ease of comparison of different cells it is appropriate to look at a small, spatially homogenous rise in calcium across the whole cell. A small rise in calcium will not saturate any of the cell's buffers, eliminating a potential problem in other published work. The buffering capacity of the cell can then be stated

simply as the number of calcium ions that will be bound for every calcium ion that remains free in the cytosol.

Knowledge of the buffering capacity of particular cells is of course useful in itself, and the development of the method described here was with that in mind. However, in many ways the magnitude of the calcium change observed following cell stimulation, is only half of the story. It is equally important to know what calcium gradients are present in the cell following stimulation and how much a cell has buffered calcium in particular areas. It is highly likely that within the cell are areas which buffer large amounts of calcium efficiently (such as close to a calcium channel - whether in the plasma membrane or an internal membrane) and other areas that are relatively poor at buffering calcium.

1.1.4 Problems associated with the use of caged compounds

The method used here to raise calcium in the cell, involved the use of a caged calcium compound, nitro-phenyl EGTA (NP-EGTA) (kind gift of G. Ellis Davies). Caged calcium compounds have been used before to examine channel regulation and cell function (Morad *et al*, 1988; Landò and Zucker, 1989; Zucker, 1993) and there are a number of problems associated with their use. In particular, work on caged compounds other than the one used in these experiments indicated interactions between calcium indicator dyes and the caged compound.

Those problems can be broadly divided into three main areas.

(a) Source of excitation light

The three most popular and widely available calcium indicator dyes are Fura-2, Indo-1 and Fluo-3. Both Fura-2 and Indo-1 are ratiometric dyes, as

outlined above (section 1.1.2). Of the two, Fura-2 is the indicator more commonly used. Whereas Fura-2 has all the advantages of a ratiometric dye related to the measurement of calcium concentration, it can be inappropriate for use with most caged-calcium compounds, since it is excited by light of the same wavelength used to release calcium from the caged compound. Fluo-3 is excited by light of a much longer wavelength than is used to photolyse the caged compound, but, since there is no shift in wavelength in either the excitation or emission spectra, ratiometric measurements are impossible. Hence, monitoring the calcium concentration in cells with Fura-2 will inevitably cause release of the calcium from the caged compound, but using Fluo-3, whilst having no effect on the photolabile calcium chelators, will involve non-ratiometric measurements, and hence be liable to inaccuracies. In this study, both problems were circumvented by the use of a marker dye to allow ratiometric measurements, with the dye Fluo-3.

(b) Interactions with the calcium indicator dye

Zucker (1992) found that both Nitr-5 and DM-nitrophen, two commonly used photolabile calcium chelators, interfered with Fura-2 and Fluo-3, and that the extent of interference depended on the calcium concentration and proportion of photolysis, since the end-products of photolysis also interfered with the fluorescence reading of the dyes.

Consideration of the absorbance spectra alone of the three compounds predicts that Nitr-5 and DM-nitrophen could affect Fura-2 fluorescence signals by screening the excitation light. The emission light would be less affected since neither of the chelators absorb significant light above 500 nm. Also, the

absorbance of the chelators is not identical at 340 and 380 nm, so the calibration of the dye is made problematic through this screening effect. To add to the problems, the photolysis products of the chelators also have significant absorbance of the excitation light, but the absorbance is different from that of the unphotolysed chelator. Thus, the ratios obtained for a particular concentration of calcium, dye and chelator, depend on the level of photolysis of the caged compound. All of these interactions also depended to some degree on the concentration of calcium present.

As well as the interference of the chelators by absorbing a significant amount of the excitation light, the chelators also displayed fluorescence themselves. Nitr-5 has an excitation spectrum similar to that of Fura-2; it also shows a change in the fluorescence spectrum with the binding of calcium, in the same way as Fura-2. However, it has little fluorescence when excited at 500 nm, the excitation wavelength for Fluo-3, and so little interference occurs. DM-nitrophen, however, is far less fluorescent than either of the indicator dyes and is not likely to affect the fluorescent signals from them.

Considering the emission spectra of the chelators, since Nitr-5 has an emission spectra that peaks at about 510-520 nm (depending on calcium concentration) it is impossible to select an emission wavelength that distinguishes between the fluorescence of the indicator dyes and the fluorescence of the chelator, since the usual emission wavelength for Fura-2 is 510 nm and for Fluo-3 is 525 nm.

The emission spectra for the two photolysis products of DM-nitrophen show that one of the products has a maximum emission at 500 nm and is excited at 380 nm, also displaying a shift in the emission peak to 465 nm in high calcium

concentrations. The other product has a fluorescence spectrum that is independent of calcium concentration and that is excited at 380 nm, emitting at 420 nm. Again, this makes it impossible to select an emission wavelength that can distinguish between the photo-products of the chelator and the indicator dyes.

(c) Destruction of the photolabile compound

There are a number of problems associated with the photolysis of caged compounds. As already discussed the photo-products of the chelator may have fluorescence properties that overlap those of the indicator dye and so the extent of photolysis is be an important factor in the extent of the interference. Also, the remaining, unphotolysed chelator is still a calcium buffer. If the whole of the chelator is not photolysed, the proportion remaining must be included in any calculation determining the buffering capacity of the cell. It is also important to know the amount of calcium that is liberated from the chelator following exposure to an UV flash, to be able to calculate the change in calcium created in the cell. It is also worth noting that the UV flash may bleach the dye (both Fura-2 and also Fluo-3, depending on the intensity of the flash). In the present study the extent of bleaching of the dyes by the UV flash was measured.

(d) Rate of release from the caged compound

The rate at which the calcium is released from the caged compound is important, especially in experiments assessing the physicochemical buffering capacity of the cell. If the rate is too slow, then the simplifying assumption, that only physicochemical buffering need be considered as operating in the time taken between the flash and complete delivery of the calcium load, will be invalid. It is therefore important that the time taken for half the calcium to be released from

the parent compound ($\tau_{½}$) is as short as possible. In experiments examining the time taken to reach half maximal tension in rabbit muscle fibres after release of calcium from NP-EGTA, this time was found to be 18 ms, which implied that release of calcium from the chelator was not the rate limiting step and that $\tau_{½}$ is less than 18 ms for NP-EGTA (Ellis-Davies and Kaplan, 1994). In contrast $\tau_{½}$ for active removal of calcium from the cytosol is likely to be of the order of a second (Goldbeter, Dupont and Berridge, 1990; Miller, 1988). Therefore we can make the simplifying assumption that after the flash calcium ions released from the chelator are present in the cytosol and available for buffering and that none are removed from the cytosol by cellular pumps before physicochemical buffers have equilibrated.

1.1.5 Method of determining calcium buffering employed here

The physicochemical buffering capacity of N1E-115 neuroblastoma cells was measured through use of a compound NP-EGTA (Ellis-Davies and Kaplan, 1994). Cells were injected with a system for measuring $[Ca^{2+}]_i$ and NP-EGTA. The injection solution contained three components:

1. A visible wavelength calcium indicator - Fluo-3

This is a calcium sensitive fluorescent dye used to measure the $[Ca^{2+}]_i$. An UV-excitable dye (Fura-2, Indo-1) cannot be used since the UV excitation would activate the "caged calcium". As Fluo-3, like most visible-wavelength calcium indicators, is a non-ratiometric dye, a calcium-insensitive marker dye is co-injected. The relative fluorescences of the calcium indicator and the marker dye are used to determine $[Ca^{2+}]_i$ in the cell, using the equation

$$[Ca^{2+}]_i = Kd \frac{(R - Rmin)}{(Rmax - R)}$$

where

Kd = calcium concentration when half the dye is saturated with calcium

R = ratio of calcium indicator dye fluorescence to marker dye fluorescence

R_{min} = ratio of calcium indicator dye fluorescence to marker dye fluorescence in zero calcium solution

R_{max} = ratio of calcium indicator dye fluorescence:marker dye fluorescence in saturating calcium solution

2. A calcium-insensitive marker dye: sulforhodamine 101

This has two functions:

i) To act as a denominator for taking ratios, since it is not calcium sensitive

ii) To act as an indicator to assess the amount of injection solution inside the cell.

This is achieved by measuring the amount of fluorescence from a known concentration of dye in a known volume, where

fluorescence = K x concentration x volume

where

fluorescence is in counts per second (cps)

K = a constant with units cps m^{-3} M^{-3} (determined experimentally)

Hence, if the volume of the cells is known the concentration of the marker dye inside the cells can be calculated from knowing the fluorescence of the marker dye in the cell. Since the concentrations of the components of the injection solution are maintained as a constant, the cellular concentrations of NP-EGTA and Fluo-3 are also known.

3. NP-EGTA

Unphotolysed NP-EGTA has a Kd of 80nM (Ellis-Davies and Kaplan, 1994), and thus at resting $[Ca^{2+}]_i$ in the cell will be approximately half saturated with calcium. Photolysed NP-EGTA has a Kd of 1mM and so will not contribute toward exogenous buffering post-flash. The calcium is released upon exposure to a flash from an UV flash gun. The area of the dish affected by the flash had a diameter of about 1.5mm.

1.1.6 Model of calcium buffering proposed

At any one time a calcium ion that has entered a cell belongs to one of three pools: bound to molecules, already removed from the cytosol (having been pumped across the cell membrane or into cell organelles) or free in the cytosol. In these experiments, additional calcium buffers are added to the cell in the form of Fluo-3 and NP-EGTA. The amount of exogenous buffer added to the cell depends in these experiments on the size of the injection volume. At low volumes, the exogenous calcium buffering introduced to the cell through injection is small in comparison with the natural calcium buffering of the cell. At higher injection

volumes, the exogenous buffering from the Fluo-3 and NP-EGTA becomes dominant.

The calcium released from the NP-EGTA by exposure to an UV flash therefore has four fates initially: binding to cell constituents, binding to the introduced Fluo-3, binding to the remaining unphotolysed NP-EGTA or remaining free. At low injection volumes, the endogenous buffers of the cell predominate but at high injection volumes, the calcium released from the NP-EGTA redistributes between the added buffers, since the buffering of the cell is negligible. The extra calcium introduced into the cell will ultimately be removed from the cytosol either into internal stores or across the plasma membrane to extracellular fluid by calcium pumps.

1.1.7 Calcium-Induced-Calcium-Release

The model outlined above is valid only if the small amount of calcium released from the NP-EGTA does not initiate the release of calcium from internal stores: calcium induced calcium release (CICR). To ensure that this is the case, experiments were repeated in the presence of 200μM ryanodine. The effects of ryanodine differ in different cells: in myocytes, ryanodine opens calcium channels on the internal stores and locks them in the open position. Once the stores have been emptied in this way, CICR cannot ensue. In other cells, ryanodine blocks the channels leaving the stores full. Either way, CICR is prevented.

1.2 CALCIUM SIGNALLING

1.2.1 Methods of raising intracellular free calcium

Calcium is a ubiquitous second messenger in cells and so it is unsurprising that several methods of raising levels of free calcium within cells exist. Free calcium is kept low in the cytosol of cells. To raise the concentration, calcium is released from areas of high calcium concentration. The level of calcium outside a cell is several orders of magnitude greater than cytosolic levels, creating a huge chemical gradient, down which calcium may flow once the permeability of the plasma membrane to calcium increases. Also, within the cell are calcium stores, where again, the concentration of calcium is far higher than the cytosol. Thus, not only does opening calcium channels in the cell membrane allow the influx of calcium into the cytosol, but also, opening calcium channels on internal membranes enclosing calcium stores allows an increase in cytosolic free calcium concentration.

I shall discuss in turn the methods of increasing cytosolic calcium concentration.

- **Calcium channels in the plasma membrane**

One of the simplest methods of raising intracellular free calcium is through the opening of calcium channels in the plasma membrane. Such calcium channels can be divided as follows.

1) Voltage operated calcium channels (VOCCs). These are found in excitable tissues and changes in membrane potential induce a conformational change in the protein such that the permeability to calcium increases. They can be

distinguished by the voltages at which activation and inactivation occur, as well as by their conductance and the response to different pharmacological agents such as dihydropyridine or cadmium (Miller, 1987).

2) Agonist gated calcium channels. Binding of an agonist to its receptor directly opens the channel. A familiar example from this class is the nicotinic acetylcholine receptor, which, on binding acetylcholine increases the membrane permeability to cations, including calcium. Expression of the α-7 receptor subunit in Xenopus oocytes showed that α-7 nicotinic receptors were highly permeable to divalent cations, especially calcium (Suguela *et al*, 1993; Sands, Costa and Patrick, 1993).

3) Second messenger operated calcium channels (SMOCCs). Plasma membrane calcium channels may also be regulated by levels of second messengers such as cAMP (Denning, Clark and Welsh, 1994) and arachidonic acid or a metabolite of arachidonate (Williams, Walsh and Doherty, 1994; Williams *et al*, 1994). Work on both excitable and non-excitable cells has shown that activation of PLA_2 and/or the production of arachidonic acid (AA) can activate calcium channels. Williams and colleagues (Williams, Walsh and Doherty, 1994) demonstrated that AA promoted calcium influx across the cell membrane through N- and L-type, voltage operated calcium channels. Murthy and colleagues (Murthy, Kuemmerle and Maklouf, 1995) found that AA, produced by the activation of PLA_2 through a pertussis toxin sensitive G-protein, mediated calcium influx through nifedipine- and verapamil-sensitive calcium channels. There may also be direct coupling of calcium channels to guanosine triphosphate (GTP)-binding-proteins (G-proteins) linking agonist-receptor

activation and calcium channel activity (Zheng *et al*, 1993; Wilding, Womack and McCleskey, 1995; reviewed by Hescheler and Schultz, 1993).

4) Capacitative influx. Depletion of $InsP_3$-sensitive intracellular calcium stores leads to the plasma membrane capacitative calcium entry (Verjans, Petersen and Berridge, 1994; DeLisle, Mayr and Welsh, 1995). Influx may also trigger influx of sodium across the plasma membrane, via a tyrosine kinase-dependent pathway (Tepel, Wischniowski and Zidek, 1994; Tepel *et al*, 1994). The nature of the signal that couples the depletion of the calcium store to the activation of calcium influx across the plasma membrane is still hotly debated. There are theories proposing a direct conformational coupling between the store and the plasma membrane. Consistent with this is the finding by Holda and Blatter (1997) that an intact cytoskeleton is necessary for capacitative influx. Other theories propose the involvement of membrane associated enzymes and/or diffusible factors. Suggested mechanisms involve the hydrolysis of GTP and may involve a small G-protein (Bird and Putney, 1993; Fasolato, Hoth and Penner, 1993) and/or the stimulation of protein kinases (Petersen and Berridge, 1995). Protein kinase C (PKC) which potentiates influx at low levels of activation, yet inhibits calcium entry at high levels of PKC activation has also been implicated (Petersen and Berridge, 1994). Work examining Xenopus oocytes suggested a role for calcium/calmodulin-dependent protein kinase II (CaMKII) in the regulation of store-depletion-evoked calcium entry (Matifat *et al*, 1997). In human mesangial cells, influx is under the control of both calmodulin and PKC (Mene, Pugliese and Cinotti, 1996). Much work has also examined the role of cyclic guanosine monophosphate (cGMP) and nitric-oxide synthase (NOS). Depletion of calcium stores activated NOS to generate

cGMP and regulate influx in pancreatic acini (Xu *et al*, 1994) and human colonic epithelial cells (Bischof *et al*, 1995) and cGMP was implicated in the modulation of a dihydropyridine sensitive calcium entry mechanism in rat pituitary GH3 cells (Willmott, Asselin and Galione, 1996). However, other laboratories found that cGMP was not responsible for calcium influx in Jurkat T-lymphocytes (Bian, Bird and Putney, 1996) and that cGMP changes are downstream of, rather than causative for, calcium influx in pancreatic acini (Gilon *et al*, 1995). Research examining the relationship between phospholipase A_2 (PLA_2) activity and calcium influx in rat thyroid FRTL-5 cells suggested that the calcium-induced PLA_2 activation and metabolites of the AA produced by a non-cyclooxygenase pathway may be implicated in the maintenance of calcium entry after the release of calcium from intracellular stores (Törnquist, Ekoski and Forss, 1994). Consistent with this, is the finding that EGF could increase the expression of 12-lipoxygenase mRNA in A431 cells (Chang *et al*, 1993), and that low levels of AA were mobilised in differentiated U937 cells following release of calcium from intracellular stores, prior to capacitative influx of extracellular calcium (Rzigalinski, Blackmore and Rosenthal, 1996), suggesting cPLA2-mediated AA release may participate in the regulation of intracellular free calcium levels. It is possible that different cell types use different methods to relate store depletion with influx across the plasma membrane.

- **Channels in the endoplasmic reticulum**

There are two major calcium channels on the internal calcium stores in a cell - ones that open in response to inositol 1,4,5-trisphosphate ($InsP_3$), and ones that respond to ryanodine/cyclic adenosine diphosphate (cADP) ribose.

1) $InsP_3$ receptor. $InsP_3$ is produced via plasma membrane phospholipid hydrolysis catalysed by the family of phospholipase C enzymes (see section "Generation of $InsP_3$" below). $InsP_3$ then diffuses away from the plasma membrane and interacts with its receptor on internal calcium stores. A family of $InsP_3$ receptors ($InsP_3R$) exists both through separate genes for the receptor protein and alternative splice variants of the genes. Calcium is released from the internal store following binding of $InsP_3$ with the receptor. The sensitivity of the $InsP_3R$ may be affected in two ways - it may depend upon the level of calcium within the store - how filled the store is - and also on variations in the receptor (Nunn and Taylor, 1992; Burgess *et al*, 1991). This could result from the existence of alternative splice variants or gene products within the same cell or population of cells, or through the phosphorylation of the receptor. Phosphorylation of the neural version leads to a decrease in sensitivity, whereas the opposite occurs in the periphery (Burgess *et al*, 1991). Bezprozvanny and colleagues found that the sensitivity of the $InsP_3$ receptor showed a bell-shaped dependence on the concentration of calcium at the cytosolic aspect of the channel (Bezprozvanny, Watras and Ehrlich, 1991). The open probability of the channel was greatest at a cytosolic calcium concentration of 300nM ($InsP_3$ concentration of 2μM). Calcium induced calcium release (CICR) may occur as the release of calcium from the store creates a localised area of increased calcium concentration close to the

receptor, such that the open probability of the channel increases, allowing a greater efflux of calcium from the store.

2) Ryanodine receptor. There is considerable structural and functional homology between the $InsP_3$ receptor and the ryanodine receptor (RyR). Again there is a family of ryanodine receptors. The receptor is named after the plant alkaloid, ryanodine, that opens the channels at low concentrations, but closes them at higher doses. The channels may also be opened by caffeine, although this aspect is complicated by the fact that caffeine may also inhibit the $InsP_3$ receptor. Ryanodine is not produced in cells as a second messenger, and the nature of the physiological trigger for the opening of these channels is the subject of considerable debate and research. The role of the RyR in CICR in excitable cells indicates that the receptor is sensitive to intracellular levels of calcium.

A candidate for the physiological ligand for the non-skeletal muscle form of the RyR is cyclic adenosine diphosphate-ribose (cADP-ribose), a metabolite of nicotinamide adenine dinucleotide. This molecule was shown to be important in calcium signalling in sea urchin eggs and has since been implicated in a number of cell types including pancreatic β cells and rat lacrimal acinar cells (Gromada, Jørgensen and Dissing, 1995a,b; Willmott, Galione and Smith, 1995), although its role is somewhat controversial. cADP ribose may have a position in intracellular signalling akin to that of $InsP_3$, but as yet there is little concrete evidence to support as ubiquitous a role as for $InsP_3$ (reviewed by Galione, 1994). RyR activation, like the $InsP_3$-R, follows a bell-shaped curve although the open probability of the channel only falls at cytosolic calcium concentrations of greater than 10μM (Bezprovzanny, Watras

and Ehrlich, 1991). Perhaps because of this difference, activation of ryanodine receptors seems often to lead to a large release of calcium, whereas $InsP_3$-R activation causes a relatively slow trickle of calcium release (discussed in a meeting report by Murphy, Poll and Westwick, 1995).

- **Generation of $InsP_3$**

$InsP_3$ is generated through the hydrolysis of a plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2) which is catalysed by phospholipase C (PLC). The action of $InsP_3$ with its receptor on intracellular calcium stores has already been discussed. The other product of phospholipid hydrolysis is diacylglycerol (DAG) which activates protein kinase C (PKC). PKC then phosphorylates specific cellular proteins leading to cellular activity and mitogenesis (see section "Protein kinase C").

A family of PLC isozymes exist, classed according to the method of their activation. I shall discuss the activation of the two PLC isozymes involved in the signalling pathways examined in this thesis.

1) Seven transmembrane segment receptors

The β isozyme of PLC is activated by the heterotrimeric GTP binding protein (G-protein) G_q (and perhaps also G_o). The receptors that activate G proteins and hence PLCβ belong to a family characterised by having seven membrane spanning domains linked by intracellular and extracellular loops. The intracellular loops interact with the G-protein. The G-proteins involved in signal transduction through these so-called heptahelical receptors are made up of three subunits, α, β and γ. When the G-protein is inactive, GDP is bound to the α subunit, and the α

subunit is complexed with the β and γ subunits. Binding of the agonist to the heptahelical receptor produces a conformational change that is transmitted to the G-protein through the cytoplasmic loops and which causes the G-protein to exchange GDP for GTP, thus activating the G-protein. The Gα.GTP complex then dissociates away from the Gβγ subunit. Both the Gα.GTP complex and the Gβγ subunit can cause activation of different PLC isozymes. Interaction of the Gα.GTP complex with PLCβ increases the GTPase activity of the α subunit, so that GTP is hydrolysed to GDP. The Gα.GDP complex then recombines with the Gβγ subunit, completing the off switch. Pertussis toxin (from *Bordatella pertussis*) ADP-ribosylates the α-subunits of some G-proteins (although G_q is not one of them), preventing the release of GDP and locking Gα in the inactive state. In A431 cells, calcium increases can be elicited by bradykinin acting through the G_q pathway (Tilly, 1987).

2) Receptor tyrosine kinases (RTKs)

PLCγ is activated through interaction with a receptor tyrosine kinase. In this case, there is direct interaction between the receptor and PLC. All RTKs comprise a ligand-binding site in the extracellular domain, a single membrane spanning helix and a cytosolic domain that possess tyrosine kinase activity. Binding of the ligand to the receptor causes most RTKs to dimerise and tyrosine phosphorylate the dimer partner on the cytosolic domain. The resulting phosphotyrosines function as binding sites for proteins containing Src homology 2 (SH2) domains. PLCγ is one SH2 domain bearing enzyme that is activated, producing $InsP_3$ and DAG. The mechanism by which PLCγ is activated is not clear, although it has

been suggested that tyrosine phosphorylation converts it to an active form (Rhee and Choi, 1992). In unstimulated cells, PLCγ is largely cytosolic but interaction of its SH2 domain with receptor tyrosine kinases induces translocation to the plasma membrane where it hydrolyses its substrate. Casabiell, Pandiella and Casaneuva found that EGF-R signal transduction was altered by *cis*-unsaturated fatty acids at some point downstream of ligand-receptor binding and autophosphorylation (Casabiell, Pandiella and Casaneuva, 1991). Both release of calcium and calcium influx in response to EGF were impaired following incubation of the cells with oleic acid.

In rat hepatocytes, EGF stimulates PLC to generate $InsP_3$ and DAG from PIP_2, and also elicits some of the tyrosine kinase activity of the receptor. The production of $InsP_3$ can be blocked by pertussis toxin but the tyrosine phosphorylation of PLCγ is unaffected, indicating the involvement of G-protein(s), but not PLCγ in $InsP_3$ production in these cells. This is unusual since the receptors that are G-protein linked that have been cloned are generally members of the seven-transmembrane spanning family of proteins, whereas the EGF receptor is a single-membrane spanning protein (Liang and Garrison, 1991, 1992).

In A431 cells, intracellular calcium concentration increases can be elicited by epidermal growth factor acing through the SH2 activation of PLCγ pathway (Meisenhelder *et al*, 1989).

Many other cellular proteins have SH2 domains and the specificity or otherwise of particular RTKs for downstream effectors is only just now being

worked out. One pathway which is particularly important links RTKs through several intermediary proteins to cell division. Binding of, say, EGF to its receptor causes receptor-receptor dimerisation and activation through autophosphorylation on tyrosine residues. Grb2, a cytosolic protein, bears one SH2 domain and two SH3 domains. The SH2 domain in Grb2 binds to a phosphotyrosine on the activated RTK and the two SH3 domains interact with another cytosolic protein, Son of sevenless (Sos). Sos becomes located to the plasma membrane and can bind to membrane bound Ras, a member of the GTPase superfamily. Grb2 therefore acts to present Sos to Ras. Ras proteins play a central role in transducing signals from RTKs to a cascade of serine/threonine kinases involved in cell differentiation and growth. Cycling of Ras between its active (GTP-bound) and inactive (GDP-bound) forms is mediated by Sos, which functions as a guanine nucleotide exchange factor, and another protein called GTPase activating protein (GAP) (Gale *et al*, 1993; Rozakis-Adcock *et al*, 1993; Li *et al*, 1993; Egan *et al*, 1993).

Activated Ras binds to the N-terminal domain of Raf, a serine/threonine kinase, which then binds to and phosphorylates mitogen activated protein (MAP) kinase kinase (MAPKK, also known as MEK). MAPKK phosphorylates tyrosine and serine residues in the protein MAP kinase, another serine/threonine kinase, activating it. MAP kinase then goes on to phosphorylate many proteins, including nuclear transcription factor involved in cell-cycle and differentiation (reviewed by Avruch, Zhang and Kyriakis, 1994).

1.2.2 Targets for the calcium signal

Changes in cytosolic calcium concentration produce a range of cellular responses, mediated by both calcium-binding proteins such as calmodulin, and phosphorylation cascades through the action of protein kinase C. I shall discuss briefly these two areas.

1) Calmodulin and other EF-hand proteins

Calmodulin is one of a family of calcium-binding proteins, termed EF-hand proteins, which binds four calcium ions in a co-operative manner. Ca^{2+}-calmodulin can then bind to and activate many intracellular proteins. A familiar example of an enzyme activated by Ca^{2+}-calmodulin is cAMP phosphodiesterase, the enzyme that promotes hydrolysis of cAMP to AMP. Other examples of EF-hand proteins include troponin C and parvalbumin.

2) Protein kinase C

Hydrolysis of PIP_2 produces two products - $InsP_3$ whose actions have been discussed, and DAG which remains membrane associated. DAG activates a family of protein kinases - protein kinase C (PKC). In unstimulated cells, PKC is predominantly cytosolic and inactive. A rise in cytosolic calcium concentration causes PKC to becomes plasma membrane associated, bringing it into close proximity to DAG which then activates it. Transient PKC activation through this mechanism leads to early responses in cells, such as the phosphorylation and thus inactivation of glycogen synthase in liver cells, promoting glycogen breakdown. Chronic PKC activation, either through the action of pharmaceutical compounds such as the phorbol esters, which mimic the activation by DAG but which are metabolically stable, or physiologically, through hydrolysis of

phosphatidylcholine (reviewed by Asaoka *et al*, 1992), leads to long-term responses such as cell proliferation and differentiation.

1.2.3 The role of calcium signalling in cell proliferation

Growth and cell division are tightly regulated in untransformed cells and free cytosolic calcium concentration changes have been strongly implicated in this regulation. The signalling pathways triggered following growth factor stimulation of cells provoke raised intracellular free calcium levels, and calcium signals seem important, both at the G_0/G_1 transition of the cell cycle and also late in G_1 (reviewed by Berridge, 1995). Calcium is involved in the regulation of immediate early genes and a maintained influx of calcium across the plasma membrane during G_1 is necessary in a number of cell types for proliferation. The activation of potassium channels leading to membrane hyperpolarisation appears to be responsible for this persistent influx of calcium. The G_0/G_1 transition signals may be linked to the differences in receptor-tyrosine kinase activity observed at different points in the cells cycle. Newberry and Pike (1995) examined the variations in cell responses to EGF in A431 cells during the cell cycle, and found that the ability of EGF to stimulate receptor-tyrosine kinase activity and phosphatidyl inositol 3 kinase (PI-3 kinase) activity varied over the cell cycle. The variation was not due to a decrease in the binding affinity of the receptor for EGF or the number of EGF-Rs on the cell surface. Some aspect of EGF-mediated signalling, downstream of EGF binding appears to be regulated during progression through the cell cycle. This is entirely consistent with the fact that once cells have

progressed through S-phase, they are committed to cell division and so responding to growth factors which provoke division is less relevant during G_2/M, than G_0/G_1.

Further evidence comes from experiments on UMR 106-01 osteogenic sarcoma cells. Bizzarri and Civitelli (1994) examined the response of cells at the G_1/S boundary to parathyroid hormone and found that a large percentage of cells in S phase responded with pertussis sensitive calcium transients to parathyroid hormone but a low percentage responded to foetal calf serum (2%). A high proportion of the cells in G_1 phase responded to 2% foetal calf serum with a pertussis insensitive calcium transients, but response to parathyroid hormone was less frequent, indicating that the calcium responses of these cells to hormonal stimulation is dependent on cell cycle.

This thesis addresses some of the receptor-mediated calcium signalling in wild type A431 cells and A431 cells expressing annexin VI. In the wtA431 cells, the interaction of two different ligands on the calcium signalling was explored. The remainder of this section will concentrate on signalling in wtA431 cells and the interactions of bradykinin and EGF on this cell line.

1.2.4 A431 cells

A431 cells are a human epidermoid carcinoma cell line, which originate from an epidermoid carcinoma from an 85 year old female (Giard *et al*, 1973). The cells over-express the EGF receptor (1-2 x 10^6 receptors/cell) in comparison with normal cells (Fabricant, De Larco and Todaro, 1977), probably through gene amplification (Merlino *et al*, 1984). As such, it is a useful cell line to examine the

mechanisms involved in signal transduction involved in the response to EGF. Much of the information examining the activation of phospholipase C by EGF binding to the EGF receptor has been obtained from these cells (Meisenhelder *et al*, 1989; Wheeler, Goodrum and Sachs, 1990; Liang and Garrison, 1992; Chen and Lin, 1993).

1.2.5 EGF receptor and signal transduction in A431 cells

Work on A431 cells has shown that cell proliferation is stimulated by pM levels of EGF, but inhibited by nM levels. This biphasic effect is not seen in normal human epithelial cells. Work by Konger and Chan (1993) indicated that 10nM EGF induces terminal differentiation of A431 cells and thus inhibited proliferation. This effect is one of long term stimulation of the cells. However, over the short-term, this level of EGF produces a spike of raised intracellular $[Ca^{2+}]_i$ followed by an elevation of $[Ca^{2+}]_i$ above basal levels (Wheeler, Goodrum and Sachs, 1990). It is this short-term signalling that is examined in this thesis and the events occurring in the first few minutes following ligand-receptor binding which shall be discussed.

Activation of the epidermal growth factor (EGF) receptor elicits several intracellular signals. The primary event that follows agonist-receptor activation is tyrosine phosphorylation of specific cellular proteins. Other events following receptor activation include activation of phospholipase C, leading to generation of two second messengers, $InsP_3$ and diacylglycerol from phosphatidylinositol bis-phosphate (PIP_2); and direct calcium-channel opening in the plasma membrane (Mozhayeva, Naumov and Kuryshev, 1990; Pandiella *et al*, 1987).

Which source of calcium is responsible for the rise in intracellular Ca^{2+}?

Since an increase in intracellular calcium can arise from both calcium entry across the plasma membrane and via release from intracellular stores following the production of $InsP_3$, which mechanism is predominant in these cells? Work by Wheeler, Goodrum and Sachs (1990) indicates that the biphasic response to EGF in A431 cells is produced by the sequential operation of the two mechanisms of calcium elevation. The initial, rapid rise of intracellular calcium is predominantly via release from intracellular stores as a direct result of $InsP_3$ production. This initial rise is not affected by the presence of the influx blocker La^{3+}. However, the long plateau of elevated intracellular calcium is absent in experiments performed in calcium-free media or in the presence of La^{3+}, indicative of calcium entry across the plasma membrane. The manganese quench technique, which allows estimation of the activity of the calcium influx pathway confirms this model. The initial rapid rise of calcium following EGF application is not associated with an increase of Mn^{2+} influx, but during the plateau phase, Mn^{2+} influx is elevated (Wheeler, Goodrum and Sachs, 1990).

Most cells have mechanisms for triggering an influx of calcium to sustain a response after the initial release of calcium from intracellular stores. The mechanism of this influx varies widely between different cells and is the subject of much research and debate.

Pandiella and co-workers (Pandiella *et al*, 1987) demonstrated that the response of A431 cells to EGF had two phases, but could not determine the method by which calcium channel activation was occurring.

Evidence for the involvement of G-proteins mediating between the EGF receptor and a plasma-membrane calcium channel, comes from patch clamp experiments (Mozhayeva, Naumov and Kuryshev, 1990) where current was measured in cell-attached and inside-out patches. Results indicated a ten-fold increase in the channel-open probability when the patches were exposed to the non-hydrolysable form of GTP, GTPγS, in a manner similar to exposure of the cells to EGF. However, few other studies have been able to confirm this hypothesis.

Many features of PLA_2 activity and AA production have been found in A431 cells. Research by Peppelenbosch and colleagues (Peppelenbosch *et al*, 1992) examined the mechanisms underlying the calcium influx in A431 cells following stimulation with EGF. They proposed a link between activation of PLA_2 leading to AA production and the calcium influx responsible for the maintained plateau of raised calcium, via a metabolite of AA, leukotriene C_4 (LC_4). LC_4 activates a calcium channel allowing a localised calcium "hot spot" to form. A calcium-sensitive potassium channel is then activated and the resultant membrane hyperpolarisation stimulates the opening of a second calcium channel. Influx through this channel produces the plateau of increased calcium concentration in A431 cells. Elements of the system proposed for A431 cells operate in other cells. Potassium channels have been suggested in other cell types to be important for a maintained, mitogenic calcium influx.

Fig 1.2.4 is a cartoon outlining the current hypothesis of the mechanism linking receptor activation and calcium influx in A431 cells.

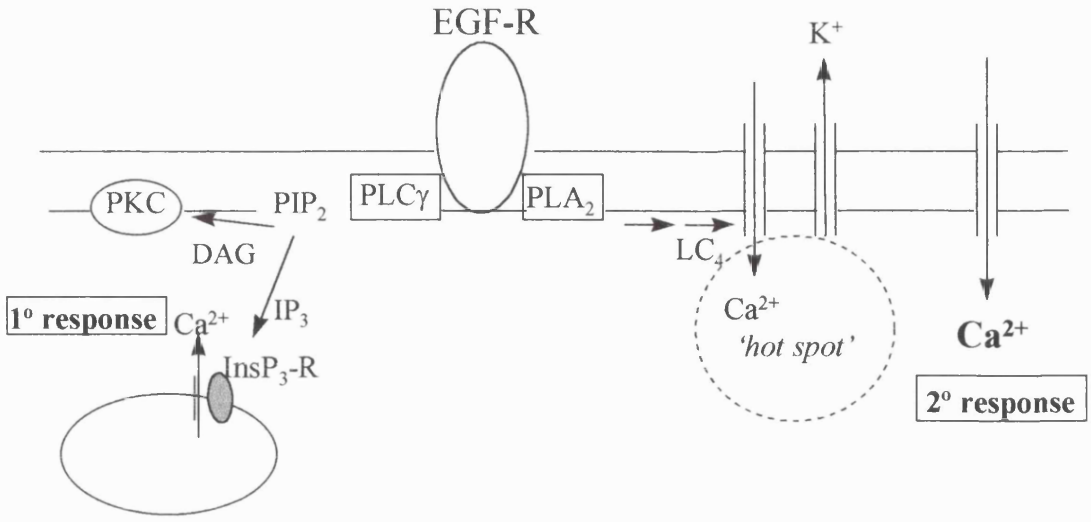


Figure 1.2.4: Binding of EGF to the EGF-R induces two separate pathways leading to the elevation of intracellular free calcium.

(1) Activation of PLCγ by tyrosine phosphorylation leads to the hydrolysis of PIP_2 to DAG and IP_3, which go on to activate PKC and the release of calcium from intracellular stores respectively. This release of calcium from the intracellular stores is the primary calcium response of A431 cells to EGF.

(2) Activation of PLA_2 causes an increase in the levels of AA. AA is metabolised in a series of steps to leukotriene C_4 (LC_4) which then activates a calcium channel. Influx of calcium across the plasma membrane then causes a "hot spot" of high calcium just beneath the plasma membrane, stimulating efflux of potassium from the cell. The resultant hyperpolarisation of the plasma membrane causes the opening of a second type of calcium channel. It is the influx of calcium through this channel that produces the secondary calcium response to EGF in A431 cells. Other factors affecting influx are sphingolipids, which in A431 cells, markedly enhanced EGF-evoked calcium influx, but had no effect on calcium release from intracellular stores (Hudson *et al*, 1994).

1.2.6 Bradykinin

Bradykinin (BK) is generated in inflammation in various tissues. In intracellular signalling systems, BK has been shown to increase the cellular concentration of cAMP and cGMP, mobilise intracellular calcium and hydrolyse phosphoinositides. It has also been reported to stimulate DNA synthesis and cell division via an $InsP_3$-mediated pathway distinct from that of EGF (Tilly *et al*, 1987).

Most of the work examining the role of BK signalling and the effect of BK on other factors stimulating cells has concentrated on long term alterations in growth patterns. Work by Kawase, Orikasa and Suzuki (1993) demonstrated that in a cloned dental pulp-cell line, BK appeared to stimulate cAMP, inositol phosphates and the calcium-signalling pathway via the generation of cyclooxygenase product(s), especially PGE_2 or a PGE_2-like compound, but that these responses did not lead to cell proliferation.

Another role for a cyclooxygenase product in BK actions is implicated in the ability of BK to prevent the proliferation that normally occurs when growth arrested normal rat kidney (NRK) fibroblasts are treated with retinoic acid and/or TGFβ in the presence of EGF. BK reduces the up-regulation of EGF-R that normally occurs, possibly by stimulating the formation of PGJ_2 (Lahaye *et al*, 1994).

McAllister and co-workers (McAllister, Leeb-Lundberg and Olsen, 1993; McAllister *et al*, 1995) examined EGF- and PDGF-induced DNA synthesis in human fibroblasts and the method by which BK inhibited it. Again, prostaglandins, particularly PGE_2 are involved although some role for cAMP is

suggested. Importantly, the differences seen in the action of BK - in some cells BK enhances growth-factor induced DNA synthesis, in others, BK inhibits DNA synthesis induced by growth factors - may be the result of the cell types studied. McAllister *et al* (1995) discuss the correlation between positive effects of BK on DNA synthesis and transformed or immortalised cell lines. The majority of work showing inhibition has been performed on normal cells. Hence, extrapolation of data from one cell type to another may be inappropriate.

Looking therefore at the work involving BK in A431 cells, Sawutz *et al* (1992) examined the interactions between TNFα and BK in A431 cells and found that the BK-receptor subtype present in A431 cells is B_2. Incubation of A431 cells with TNFα causes a decrease in BK-R number, but not affinity, probably through a G_1 arrest causing reduced DNA synthesis. Since there is a constant turnover of receptors, a reduction in new receptor production would cause a decrease in BK-R number without affecting affinity.

It is clear from this short summary of the long-term effects of BK on cells that BK can promote many intracellular signalling pathways and that interactions between these pathways must occur. Although most of the work done so far has concentrated on the long-term effects of BK on cell function, there is obviously some scope for a short-term interaction between receptors and/or signalling pathways. This thesis examines the interactions of BK and EGF on A431 cells in short term calcium signalling - whether there is some form of cross-talk between the receptors and their pathways.

1.2.7 Cross-talk between receptors

There may be a significant degree of interaction between the receptors that activate PLC. In A431 cells, bradykinin is thought to cause phosphorylation of the EGF receptor via PKC. This phosphorylation has the effect of reducing the binding of EGF to the high-affinity binding site on the receptor (Hosoi, Kurihara and Ueha, 1993). Since the difference between the concentration of EGF needed to induce calcium oscillations in the cell and the concentration of EGF required to produce the peak-plateau response is very tight (Cheyette and Gross, 1991), even a small shift in the dose response curve caused by phosphorylation of the receptor could have significant effects on calcium signalling in the cell.

Cheyette and Gross (1991) reported that the response to EGF of A431 cells has three manifestations: at high concentrations of agonist, the cells respond with a rapid elevation of calcium followed by a plateau of raised cytosolic calcium - the peak-plateau response. At low concentrations, the cells undergo calcium oscillations, with no raised plateau. At intermediate concentrations, the cells show a peak that subsides back to resting levels with little or no plateau.

PKC is activated by calcium and DAG, and so the effect on the phosphorylation state of the EGF-R, could in principle be caused through increased levels of DAG (from hydrolysis of PIP_2) or as a result of a rise in intracellular free calcium.

The potential for complex interactions between the various signalling pathways is clear, although no research has previously been conducted into manifestations of these interactions in the whole cell. In this work, I have

examined whether prior exposure to EGF affected the calcium response of A431 cells to a subsequent challenge with BK and *vice versa* in intact cells.

1.3 ANNEXIN

1.3.1 The annexins: A family of proteins

The annexins are a family of proteins characterised by calcium dependent phospholipid binding (Evans and Nelsestuen, 1994). The role of annexins is at present very unclear: many roles for this family of proteins have been suggested, including involvement in exocytosis, channel formation, inhibition of phospholipase A_2, membrane-cytoskeletal linkage and regulation of cell surface receptors (Zaks and Creutz, 1991; Kaetzel and Dedman, 1995; Demange *et al*, 1994). All annexins share a seventy amino acids domain that in annexin VI appears eight times, but in all other annexins appears four times. The amino terminal domains of annexins are very variable in both length and amino acid composition. It is suggested that region is responsible for the different functions of the annexins.

Functions of the annexins:

Before addressing the proposed actions of annexin VI specifically, it may be worth mentioning briefly some of the properties put forward for some of the other members of the family.

It is somewhat paradoxical that a family of proteins with so much conservation within the family has been proposed to have such a wide range of functions. A further complication is that functions apparent *in vitro* may not be reflected *in vivo*, in much the same way as was found for calmodulin. As with calmodulin, the annexins may have different actions in different cell types and

many of the properties so far described *in vitro* for the various members of the family may be found to have no physiological relevance.

In general, the annexins can be described with the following properties. On the addition of calcium, or an increase in calcium intracellularly, they shift from a soluble form to a membrane-associated form (Evans and Nelsestuen, 1994). The monomers join up to form arrays in the membrane around target proteins (Zaks and Creutz, 1991). This alters the fluidity of the membrane and also the distribution of phospholipids in the plasma membrane, possibly altering membrane functions (Kaetzel and Dedman, 1995). The membrane becomes stabilised and phospholipid-binding proteins such as PKC, and cellular phospholipases such as PLA_2 may be inhibited or hindered from becoming membrane-associated.

In a review of the actions of annexins in 1989, Burgoyne and Geisow suggested that there were three main roles for the annexins - exo- and endocytosis, attachment of cytoskeletal elements to the plasma membrane and inhibition of PLA_2. The last, along with some other features subsequently described in the literature, is likely to be an *in vitro* artefact caused by the sequestration of calcium and/or phospholipids by the annexins.

Generally, a multitude of actions for each annexin have been proposed. As a welcome exception to this, annexin III has been identified as the enzyme inositol 1,2-cyclic phosphate 2-phosphohydrolase (Ross, Tait and Majerus, 1990). The functions of other members of the family however are less resolved. As well as the features mentioned in Burgoyne and Geisow's review, various members of the group of proteins have been described as calcium channels, substrates for phosphorylation by EGF and PKC and inhibitors of PKC.

Lipocortin I (annexin I) has been shown to be distributed not only in cytoplasm and at the plasma membrane in cells, as is generally found with the annexins, but also in the nucleus of endothelial cells (Raynal *et al*, 1992). The same protein has been said to be phosphorylated by the EGF-R (Sawyer and Cohen, 1985; Pepinsky, 1991) and is a good substrate for phosphorylation by PKC (Schlaepfer and Haigler, 1988). It is also involved in the anti-inflammatory response provoked by glucocorticoids (reviewed by Goulding and Guyre, 1993 and Flower and Rothwell, 1994) although it is still unresolved whether the action is intra- or extracellular, or both.

Annexin V has been claimed to be able to form ion-selective, voltage gated channels in membranes (reviewed by Demange *et al*, 1994) although arguments against this include the fact that annexins do not penetrate into the membrane, but remain peripherally bound. In answer to that criticism, it has been suggested that annexin V can provoke the formation of membrane pores - "the microscopic electroporation phenomenon". Work by Goossens *et al* (1995) found that annexin V disrupted membranes at low calcium concentrations, but stabilised them at higher calcium concentrations. Calcium was not transported into artificial vesicles containing the calcium indicator dye Fura-2 and they deduced that annexin V is unlikely to be a calcium channel. Both sides of the argument are based on *in vitro* studies. Other *in vitro* studies on annexin V indicate that it inhibits PKC, and rule out the possibility of this being secondary to the calcium and/or phospholipid sequestering feature of the protein (Schlaepfer, Jones and Haigler, 1992; Shibata, Sato and Maki, 1992). Shibata, Sato and Maki also found that annexins VI, III and a mixture of I and II inhibited PKC phosphorylation of histone.

1.3.2 Annexin VI

Turning attention to annexin VI, the member of the annexin family examined in this thesis, again, several functions have been proposed, with no apparent common theme linking the properties. As already mentioned, *in vivo* and *in vitro* results often differ, and a function discovered in a cell free system may frequently not be replicated in the cell. I will briefly review what is known.

Biochemical properties of annexin VI:

Whereas all the other annexins have four repeats of a domain of approximately 70 amino acid residues, annexin VI has eight repeats. Analysis of the residues indicates that the greatest similarities between the domains are between 1 and 5, 2 and 6 et cetera. It would appear that annexin VI has arisen through duplication of a four-repeat annexin or fusion of two four-repeat proteins (Smith and Moss, 1994). There are two splice variants one of which includes an exon of six amino acid residues, so that annexin VI usually appears as a closely spaced doublet in SDS-polyacrylamide gel electrophoresis. It is reported to have one binding site for calcium with a Kd of 1.2μM in the absence of phospholipid and eight calcium-binding sites in the presence of phospholipid. Zaks and Creutz (1991) report evidence for calcium-dependent self-association of annexin VI, with the level of calcium dependency altered by membrane phospholipid composition, such that self-association may occur at more physiological calcium levels.

Functions of annexin VI:

Annexin VI has been implicated in both endo- and exocytosis since, like all the annexins, it binds phospholipids in a calcium dependent fashion (Lu, Bazzi and Nelsestuen, 1995). The protein is predominantly expressed in endocrine cells, with the exception of the parathyroid (Clark *et al*, 1991), and so a link with secretion seems plausible. However, as ever, caution must be employed when attempting to extrapolate from *in vitro* assays to functions of the protein *in vivo*. In work examining the role of annexin VI in endocytosis, Lin and colleagues (Lin, Südhof and Anderson, 1992) looked at the formation of coated vesicles from coated pits in a cell free assay. Coated vesicle formation from coated pits requires ATP (possibly for phosphorylation) and calcium (for membrane-membrane fusion) and a cytosolic factor. If annexin VI was removed from the cytosolic element in the assay, coated pit budding ceased and the re-inclusion of purified annexin VI restored activity. Purified annexin VI on its own does not have full activity, so there is presumably some other cytosolic factor that is required for full activity. However, work on A431 cells, a cell used extensively to examine endocytosis which lacks annexin VI, found that the protein was not essential in the recycling of the transferrin receptor (Smythe *et al*, 1994). Indeed, the expression of annexin VI into these cells did not have any effect on the kinetics of receptor recycling, nor did the addition of purified annexin VI to permeabilised A431 cells support scission of coated pits. This difference in results may well be an aspect of the different features of the annexins *in vitro* c.f. *in vivo*.

Membrane fusion is also necessary in exocytosis and annexins have been implicated in this process too. In human neutrophils, exocytosis of granules is

triggered by a rise in calcium, and the three granule populations in neutrophils are discharged at different calcium concentrations. Sjölin, Stendahl and Dahlgren (1994) looked at the location of annexins in neutrophils under different conditions and found that annexins are present in the cytosol and translocated to the plasma membrane and also to the isolated organelles with increased cytosolic calcium concentrations, consistent with the annexins having a role in exocytosis. The role of annexins in exocytosis was reviewed by Creutz in 1992, however much of the work reviewed was from *in vitro* assays and may not be applicable *in vivo*.

Annexin VI has been shown to be able to form a *de novo* ion channel in phospholipid membranes (Pollard *et al*, 1992). Annexin VI has also been shown to be closely associated with organelles involved with the sequestration and/or release of calcium. Rainteau *et al* showed that annexin VI is located on the inner membrane of bovine liver mitochondria. (Rainteau *et al*, 1995) and suggest that this is consistent with annexin VI having a role as a calcium channel. Díaz-Muñoz and Hamilton (1990) showed that annexin VI modified the gating behaviour of the calcium-release channel on sarcoplasmic reticulum heavy vesicles inserted into an artificial membrane. It increases the open probability and mean open time of the calcium release channel. This modulatory effect was calcium-dependent and specific to the original luminal aspect of the channel. The annexin VI-modified channel remained sensitive to ruthenium red and ryanodine. Recordings from the calcium release channel in intact sarcoplasmic reticulum resemble more closely the modified channel, than the control where no annexin VI has been added. The action of annexin VI is specific to the calcium release channel, and does not affect the potassium or chloride channels from the sarcoplasmic

reticulum, nor the dihydropyridine-sensitive calcium channel from the transverse tubule.

Hazarika and colleagues (Hazarika *et al*, 1991a) confirmed that there was no effect of annexin VI on calcium uptake or release when added to the myoplasmic aspect of the sarcoplasmic reticulum, as opposed to the luminal aspect. Further work (Hazarika *et al*, 1991b) showed that the full annexin VI protein was needed to effect channel regulation and that neither the amino terminal half nor the carboxyl terminal half alone could support regulation, despite each fragment retaining calcium dependent phospholipid binding properties.

Following the theme of annexin VI in calcium homeostasis within the heart, Gunteski-Hamblin *et al* (1996) produced transgenic mice with over expression of annexin VI targeted to the heart. The mice presented with numerous pathological conditions of the heart, including enlargement, myocarditis and fibrosis of the heart. Annexin VI over-expression leads to an alteration in normal calcium dynamics and contractility. Cardiac myocytes from the transgenic mice had lower basal calcium concentrations and a decreased rise in free calcium after depolarisation. It may be that annexin VI is affecting pumps and/or exchangers in the membranes of cardiac myocytes. The impaired calcium homeostasis leads to cell death and the replacement of myocytes with fibroblasts.

Different annexins recognise secretory vesicles differently. Annexin VI was found to inhibit the aggregation of granules promoted by other annexins *in vitro* (Creutz *et al*, 1992). Annexins have also been shown to bind to chromaffin granules - a large intracellular calcium pool in adrenal cells, and so Jones, Fitzpatrick and Waisman (1994) looked at the role of annexins in chromaffin granule calcium homeostasis. They found that when annexin VI was added to a

preparation of chromaffin cells there was release of calcium from the granules, through either binding to and activating an existing mechanism or through the formation of *de novo* channels.

There are obviously strong links between annexin VI and calcium in some aspect within cells but there is no consistent result that covers many cells types or functions. There are proposed links with calcium sequestration and channels but little work on the physiology that any effect would have. Theobald and colleagues examined the effect of causing cells normally lacking annexin VI to express the protein. A431 cells do not express annexin VI but can be invoked to do so after transfection with plasmids containing the DNA for the protein. During routine passaging of the cells - both wtA431 cells and the annexin VI expressing clones, Theobald and colleagues noticed that annexin VI^+ cells had longer doubling times in low serum conditions and became growth arrested (Theobald *et al*, 1994). On further investigation, it was found that annexin VI^+ cells were growth arrested in the G_1 phase of the cell cycle when cultured in low serum conditions, whereas wtA431 cells showed the same proportion of mitotic cells in both high and low serum conditions. They concluded that annexin VI moderates cell proliferation. Another difference noticed in the cells expressing annexin VI was that they became contact-inhibited on reaching confluence, a characteristic that is absent in the wild type.

Other work by Theobald and colleagues (Theobald *et al*, 1995), has shown that annexin VI has tumour suppresser activity in A431 cells. Both control cells that did not express annexin VI and annexin VI^+ cells were injected separately into the flanks of nude mice and the size and morphology of the tumours induced compared. The tumours produced by the two cell types were morphologically

indistinguishable, but the tumours originating from annexin VI^+ cells were, on average, 40% smaller than the tumours resulting from injection of the control cells. The expression of the protein appears to diminish the growth rate of the tumour *in vivo* and the negative effects on growth observed in these cells are not an artefact of *in vitro* conditions.

Interestingly, the expression of annexin VI was found to differ in melanocytes as they progressed from benign to malignant phenotypes (Francia *et al*, 1996). Following their finding that four transcripts were differentially expressed between melanocytes and melanomas and that one of these - the transcript expressed preferentially by non-tumorigenic melanocytes - encoded for murine annexin VI, the group examined the expression of annexin VI in naturally occurring human melanomas. They found a distinct correlation between reduced expression of the protein and the tendency of the tumours towards malignancy, consistent with both the *in vitro* studies on decreased growth rates and the *in vivo* studies on tumour size. All three strands of research indicate that annexin VI is affecting cell growth in a negative fashion.

Since the growth of A431 tumours depends on the presence of EGF (Kitagawa *et al*, 1995), this thesis examined the effect annexin VI has on EGF-stimulated calcium signals in A431 cells. The calcium responses evoked in parental A431 cells were compared with those in annexin VI^+ cells to try and illuminate the method by which annexin VI cells become growth inhibited.

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Solutions

For the duration of the experiments, cells were maintained in saline as follows:

In experiments determining calcium buffering cells were maintained in either saline A or B (see table 1) (TBS or HBS respectively). For the experiments on annexin transfected cells and for the investigation into cross-talk between receptors, saline B was used. Some of these experiments used Ca:free saline - saline C (Ca-free:HBS). Experiments investigating the quenching of Fura-2 by manganese used saline D (Mn-HBS).

Table 1: Salt concentrations in salines used (all in mM)

	A	B	C	D
NaCl	120	120	120	120
KCl	5.5	5.5	5.5	5.5
CaCl2	1.8	1.8	-	1.8
MgCl2	1	1	1	1
Glucose	25	25	25	25
HEPES	-	20	20	20
TRIS	20	-	-	-
EGTA	-	-	1	-
MnCl2	-	-	-	0.5*
pH	7.2	7.2	7.2	7.2
Abbreviation	TBS	HBS	Ca-free:HBS	Mn-HBS

*made from a stock of 1M $MnCl_2$, prepared fresh daily, to avoid potential problems with Mn^{2+} oxidisation to Mn^{3+}.

Ryanodine:

In experiments with ryanodine present, a stock solution of 1mM ryanodine (Calbiochem) was made up in phosphate buffered saline (SIGMA) and added to the dish to produce a final concentration of 200μM. All measurements taken in the presence of ryanodine were made after a minimum of ten minutes.

Catalase:

A stock solution of catalase (SIGMA) was made up in water and added to the dish to a final concentration of 125u/ml.

4-OH TEMPO:

A stock solution of 50mM 4-OH TEMPO (SIGMA) (in H_20) was prepared and added to the dish to a final concentration of 500μM.

Bradykinin (BK):

For experiments in which cells were stimulated with bradykinin (SIGMA) a stock concentration of 1mM was produced by dissolving the product in phosphate buffered saline (SIGMA). Bradykinin was routinely used at 1μM.

Epidermal Growth Factor (rhEGF):

Recombinant human EGF was purchased from Promega. The lyophylised product was rehydrated in calcium and magnesium free phosphate buffered saline to produce a stock concentration of 100μg/ml rhEGF. rhEGF was routinely used at 100ng/ml, except in dose response curves. Stock was diluted down to the required final concentration in HBS. For experiments involving calcium free saline, the stock was diluted to a final concentration of 100ng/ml in calcium free saline (with 1mM EGTA present). For experiments involving Mn-HBS, the stock solution was diluted to the final concentration of 100ng/ml in Mn-HBS.

2.1.2 Injection solutions

The injection solution used in all buffering experiments consisted of 28mM Fluo-3 (Molecular Probes), 14mM sulforhodamine 101 (Molecular Probes) and 22.68mM nitrophenyl-EGTA (NP-EGTA) (kind gift of G. Ellis-Davies). Stock solutions of the components were as follows; Fluo-3: 100mM in water;

sulforhodamine: 23.7mM in 130mM KCl and NP-EGTA: 176 mM, as determined by their extinction coefficients. The injection solution will be referred to as SR/F/NP from here on. The NP-EGTA was not loaded with calcium in the injection solution and thus is loaded according to the resting $[Ca^{2+}]_i$ of the cell.

Other injection solutions:

In other experiments, cells were injected with either 10mM Calcium Green Dextran (MW of 10,000) (Molecular Probes) in 130mM KCl or 20mM Fluo-3 (Molecular Probes) in 130mM KCl.

2.1.3 AM loading of the cells

In experiments investigating UV damage to the cells, cells were AM-loaded with Fluo-3 AM (Molecular Probes). A stock solution of 2mM Fluo-3 AM plus 2.5g/100ml pluronic F-127 (Molecular Probes) was made up in DMSO and added to the medium bathing the cells to give a final Fluo-3 AM concentration of 2μM. In all the studies involving rhEGF and BK, cells were loaded with Fura-2 AM (Molecular Probes). A stock solution of 2mM Fura-2 AM plus 2.5g/100ml pluronic F-127 was made up in DMSO and added to the medium bathing the cells to give a final Fura-2 AM concentration of 2μM.

Cells were loaded with 2μM dye in the media they were cultured in and remained in a 33°C, 5% or 10% CO_2, fully humidified incubator for 30 mins. After loading, the cells were removed from the incubator and rinsed with saline (TBS or HBS as appropriate).

In experiments comparing the buffering capacity of wtA431 cells and cells expressing annexin VI, some cells were pre-loaded with BAPTA AM before

injection as follows. BAPTA AM was prepared in DMSO containing 2.5% (w/v) pluronic F-127 to a stock concentration of 30mM. BAPTA AM was used at an extracellular concentration of 40μM. Cells were incubated with 40μM BAPTA AM in the media they were cultured in and remained in a 33°C, 5% or 10% CO_2, fully humidified incubator for 30 mins. After loading, the cells were removed from the incubator and rinsed with saline.

2.1.4 Cell cultures

(a) Buffering Capacity

N1E-115 neuroblastoma cells

All cells used to determine buffering capacity were from cultures of N1E-115 neuroblastoma cells maintained in Dulbecco's modification of Eagle's medium (DMEM) (ICN Biomedicals Inc.) buffered with 1.21 g/litre $NaHCO_3$ and supplemented with 10% foetal calf serum (FCS) (Advanced Protein Products) in an atmosphere of 5% CO_2 at 33°C. Cells from these maintained cultures were plated on hydrophilic Petriperm (Bachofer, Reutlinger) dishes in the presence of 2% dimethyl sulfoxide (DMSO) (SIGMA) to induce differentiation (Kimhi *et al*, 1976). Cells were used in experiments after a minimum of three days from plating, by which time many cells had produced neurites. Only cells without neurites were used in this study. Both long term cultures and the plated cells until use were kept in a 33°C, 5% CO_2, fully humidified incubator.

Dorsal root ganglion cells

Cells used to investigate the effects of damage from the UV flashes were rat dorsal root ganglion (DRG) cells. The DRG cultures were prepared as described by Lindsay, Evison and Winter (1991). 150g male Sprague-Dawley rats were killed with CO_2 but not cervically dislocated. The spinal cord was removed and split dorso-ventrally into two halves. The dorsal root ganglia were removed and placed in 0.125% collagenase (Worthington Biochemical Corporation, New Jersey) in F14 (Imperial, UK) media for three hours, then washed with F14 media supplemented with 5% USG (Gibco, BRL) (F14USG). After washing and centrifuging, the cells were triturated in 1ml of F14USG to form a single cell suspension. This suspension was made up to the required volume with F14USG and aliquoted into hydrophilic Petriperm dishes such that there were 4 mls of cell suspension per dish. All Petriperm dishes had been coated with polyornithine (SIGMA) (5μg/ml in H_20 for 1 hour) followed by laminin (Life Sciences, New York; produced by Engelbreth-Holm-Swarm Mouse sarcoma) (5μg/ml in phosphate buffered saline (SIGMA) for 1 hour). The plated cells were kept in a 33°C, 5% CO_2, fully humidified incubator until use.

(b) Annexin VI transfected cells

Transfections:

Transfection of A431 cells with annexin VI were performed by members of Stephen E. Moss' laboratory (University College London, Gower Street, London. WC1E 6BT). Transfection of the wild type cells was with pRC/CMV and pRC/CMV.anx6. The construct pRC/CMV.anx6 contains a full length human

annexin VI cDNA which was created by the ligation of partial length clones A2 and 10.6 (Crompton *et al*, 1988). The full-length cDNA was directionally cloned into HindIII/XbaI cut pRC/CMV (Invitrogen Corp., UK). On the day prior to transfection, exponentially growing A431 cells were sub-cultured at approximately 10^4 cells/cm^2 on 90 mm dishes and cultured for an additional 24 h. Transfection was achieved using the calcium phosphate method. Neomycin-resistant colonies were isolated after two weeks and examined for expression of annexin VI by Western blotting. Subconfluent cultures in 90 mm dishes were lysed *in situ* by the addition of 1ml ice cold 10mM TRIS-HCl, 140mM NaCl, 2mM EGTA, 1% Triton X-100 and 1mM phenylmethylsulphonylfluoride (PMSF). The lysates were clarified by centrifugation and proteins were resolved by electrophoresis through 10% SDS polyacrylamide gels. The proteins were then transferred to Immobilon P (Millipore) and the membranes incubated with rabbit polyclonal anti-human annexin I or VI antisera and then with alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (1:10,000) (Promega, U.K.). Immunoreactive bands were visualised by development with Western Blue, (Promega, U.K.). Control transfectants were generated using identical procedures but with the wild-type vector.

Cell Culture:

Both wild type A431 cells and transfected cells were grown and maintained in DMEM supplemented with 10% foetal bovine serum (FBS) (Gibco, Paisley, Scotland), 100 units/ml penicillin, 0.1mg/ml streptomycin, 1% glutamine, 20µls/l PABA and 1% sodium pyruvate. Cells were plated on Petriperms (hydrophilic, Bachofen, Reutlinger) or on glass coverslips (BDH) in 90 mm dishes. Cells were

plated from confluent or sub-confluent dishes as follows: cells were washed with sterile phosphate buffered saline. Trypsin was added to each dish and the cells replaced in the incubator until the cells were lifting off the bottom of the dish. The cells were pipetted off the dish and triturated through a 5ml pipette. Final cell density was approximately 30%. Cells were used on the first day following plating, since the cultures grow rapidly and preliminary work by Dr. P. Mobbs (Dept. of Physiology, University College, London) indicated that a large variation in their response to rhEGF occurred depending on density and age of the cultures. On the first day post-subbing, the cells are still in small groups and have not reached a highly confluent state. In this study I restricted analysis to single cells together with groups of at most three cells.

2.1.5 Equipment

Microscope and measuring system for the buffering experiments

In the buffering experiments, cells were removed from the incubator, rinsed with TBS and viewed on a Zeiss WL epifluorescence microscope with either a 32x (Leica) dry objective or a 63x water immersion objective (Zeiss). The NA of the 63x objective was 1.4. The light source supplying the microscope for fluorescence excitation of the cells was an incandescent bulb driven by a current regulated power supply (Hewlett Packard). Light passed through a heat filter and the appropriate wavelength of light necessary for excitation of the two dyes (sulforhodamine 101 or Fluo-3) was selected through use of two filter cubes. The filter set used for sulforhodamine 101 was excitation 580 nm DF27, longpass dichroic at 605 nm, emission 630 nm DF30. The filter set used for Fluo-3 was

excitation 450 nm DF55, longpass dichroic at 505 nm, emission 535 nm DF35. All emission fluorescence passed to either the eyepieces of the microscope or the photomultiplier tube (PMT) (Thorn EMI), through the objective. The software used to collect emission fluorescence from the cells through the PMT was photon counting software from Thorn EMI.

Flash gun

The UV flash gun used in buffering experiments was a Hi-Tech Scientific XF-10 xenon flash lamp system. The flash was focussed by use of the objective included in the system and a quartz accessory lens. The resultant spot is finely focussed to approximately 1.5 mm in diameter. The diameter of the spot was determined using photosensitive paper (Medelec, England). Injected cells were a minimum of 5 mm apart in the dish and so the UV flash should not cause significant bleaching of adjacent injected cells. For visualisation of the cells during injection, white light was passed through a 550 nm bandpass filter to prevent photolysis of NP-EGTA. The flash produced by the gun was passed through a Schott BGI, visible block, UV pass filter, which passes 300-450 nm.

The imaging system:

The system used for imaging cells consisted of an inverted epifluorescence microscope, an ultraviolet light source, a camera and a computer for collecting information from the camera and for the analysis of the images obtained.

The epifluorescence microscope

For all the experiments examining cell signalling, a Zeiss IM microscope with a 40x glycerol immersion objective (Neofluor UV-F, Nikon) was used. The numerical aperture of the lens was 1.3. The light source supplying the UV light was fitted with a 150W high pressure xenon arc lamp (Wotan, Germany) and a KG1 heat filter. To obtain light of wavelengths 350 nm and 380 nm for excitation of Fura-2, appropriate narrow band filters were rotated into the UV path using a computer controlled stepper motor. The filtered light was then reflected using a dichroic mirror (400 nm longpass) and focussed on the cells through the objective. Emission from the cells also passed through the objective and through the dichroic and a broad band filter of centre wavelength 510 nm (Glen Spectra), before going either to the camera or the eyepieces of the microscope.

For manganese quench experiments, a Zeiss Axiovert epifluorescence microscope with Fluor objectives was used. The light source supplying the UV light was a 50W high pressure xenon bulb and wavelengths of light were selected by a monochromator (Till, Munich). The excitation light was then reflected using a dichroic mirror (400 nm longpass) and focussed on the cells through the objective. Emission from the cells also passed through the objective and through the dichroic before going either to the camera or the eyepieces of the microscope.

Data acquisition and analysis

The camera used with the imager system in the calcium signalling experiments was an intensified CCD camera (extended ISIS-M, Photonic Sciences, UK) with a greater signal to noise ratio in the blue-green range of the

spectrum. The images for analysis were obtained using a personal computer based image processing system comprised of a video adapter, a Fidelity 100 Frame Grabber board (Data Translation, MA), in a 486 DX 66 Mhz PC with 16 MB random access memory and 230MB hard disk memory. Images from the camera were digitised as an array of 768 x 576 pixels using a program written by Kevin Boone. Data was subsequently stored on 1 GB optical disks (Panasonic) and analysed with software from Global Lab Image (Data Translation) and a program written by Kevin Boone. The Till imaging system used a cooled CCD camera and proprietor's acquisition and analysis software (FUCAL, Till Photonics, Munich).

Perfusion Chamber

To effect rapid perfusion of solutions over the cells, a diamond shaped perfusion chamber was used (Warner, Hamden, Connecticut). The chamber was attached to the surface on which the cells were growing using silicone grease to ensure a water-tight seal. The solutions fed to the top of one apex of the chamber via cannulae from syringes mounted to the side of the set-up and were withdrawn from the bottom of the other apex via a suction pump.

2.2 METHODS

2.2.1 Experimental Protocols

(a) Calcium Buffering:

The following protocol was used for all the buffering experiments and was therefore performed on N1E-115 neuroblastoma cells, wild type A431 cells and annexin transfected cells.

Cells were removed from the incubator and the dishes rinsed with HEPES buffered saline (HBS). The cells were impaled under a 32x objective with a glass micropipette (resistance of approximately 15 MΩ when loaded with 2M KCl) containing at the tip SR/F/NP (section 2.1.2) solution and back-filled with 130mM KCl. Dye was ejected from the tip into the cell by applying pressure to the back of the pipette. The cells were allowed to recover for a few minutes, and the dish rinsed with fresh HBS. After recovery, the cells were monitored using a 63x objective and the longest and shortest axis of the cell measured via an eyepiece graticule. The average of these two values was calculated and the volume of the cell calculated by applying the equation $v = 4/3\pi r^3$ (where v is the volume of the cell and r is the average of the axes). Initial values of sulforhodamine and Fluo-3 fluorescence, with a neutral density filter of 1.4 present in the excitation beam, were measured in order to calculate the resting ratio of the cell and thus resting $[Ca^{2+}]_i$. The cell was then monitored measuring only Fluo-3 fluorescence with the neutral density filter of 1.4 removed from the excitation beam. At t = 60 seconds one UV flash, attenuated with a neutral density filter of 0.6 in the path of the flash, was fired at the cell. There was no break in the recording by the PMT and a

flash artefact of approximately 100 ms (see section (g) below) was present in the recordings. Fluo-3 fluorescence was monitored for approximately 5 minutes and in most cells a second ratio of Fluo-3 fluorescence to sulforhodamine fluorescence (with neutral density filters 1.4 in the path of the excitation beam) was taken to assess whether there was significant dye loss from the cell. Background readings were taken on a nearby uninjected cell of the same dimensions as the injected cell to allow estimation of cellular autofluorescence and other background signals.

In all analyses of the data, a period of 500 ms from the start of the flash artefact was removed and exponentials fitted to the data for the period 500 ms to 1500 ms after the flash. The data was extrapolated back to find the value of the Fluo-3 fluorescence immediately after the flash. Similarly, drift in the signal before the flash was removed by fitting a line to the data over 1000 ms before the flash and taking the value of this line at the moment of the flash. The data presented in the figures has not been drift-corrected.

(b) Calibration of the injection solution:

The concentrations of the components of the injection solution had to be known precisely, to be able to calculate the endogenous buffering capacity of the cell. Stock solutions of each of the components of the injection solution were calibrated using a spectrophotometer. By measuring the absorbance of a specific wavelength of light by the compound, the concentration of the stock solutions can be checked, using the equation $A=\varepsilon bc$, where A is the absorption, ε is the extinction co-efficient of the compound, b is the path length of the cuvette and c

is the concentration. This was performed on all stock solutions of the compounds before analysis of data to ascertain final cell concentrations of all components of the injection solution.

(c) Experimental determination of K:

To use the known fluorescence of the sulforhodamine in the cell to calculate the concentration of NP-EGTA and Fluo-3, the equation

Fluorescence = K x concentration x volume

was used. K was determined experimentally by measuring the fluorescence of a known volume of a solution of sulforhodamine of known concentration. The value for K was determined for each fresh batch of injection solution. I confirmed that there was no difference in the value of K with differing concentrations of calcium, that is, there was no contamination of the sulforhodamine signal by Fluo-3 fluorescence. Again, neutral density filters of value 1.4 were included in the path of the excitation beam for both the *in vitro* calibration and when recording from the cell, to bring the level of fluorescence from sulforhodamine below the level of saturation of the photomultiplier tube.

(d) Calibration of the UV flash gun:

In vitro calibrations of the flash gun proved to be unreliable and inconsistent, although no obvious reason why this should be so could be found. Results seemed to be highly dependent on spatial characteristics of the calibration system; results from solutions in droplets differed from solutions in glass microcuvettes with a path length of 50μm (Camlab, Cambridge, England). Hence, the cells were used

as an internal control. The precise model applied is described in section 3.3.1 and only the principle will be discussed here. In heavily injected cells, the endogenous buffering of the cell is masked by high concentrations of the exogenous buffers, Fluo-3 and NP-EGTA. Thus, the contribution of endogenous calcium buffering is negligible and can be excluded. The total buffering capacity of the cells in this situation is able to be calculated since the concentrations of all the exogenous calcium buffers are known, and the calcium released from NP-EGTA (and thus the intensity of the UV flash gun) can hence be resolved.

For this approach to work, the output of the UV flash gun must be consistent from day to day. This was ascertained by firing a heavily attenuated UV flash at a microcuvette containing a solution of 200μM Fura 2 in a saturating calcium solution (see (i) below, in the section on calcium calibrations) and measuring the fluorescence intensity at the moment of the flash. This fluorescence was monitored weekly and the output of the flash gun altered where necessary to maintain a constant flash intensity.

(e) UV damage in the cells:

The unattenuated UV flash damaged the cells (see Results section 3.1.3). Unless otherwise noted, the UV flash was attenuated with a 0.6 neutral density filter in the path of the UV flash. With the filter in place, no photo-damage was observed (see Results section 3.1.3).

(f) Photobleaching of Fluo-3 and SR:

Solutions of 10μM Fluo-3 or sulforhodamine were made up in 150mM HEPES, pH 7.2 and loaded into microcuvettes (Camlab, Cambridge, England). The microcuvettes were then exposed to an UV flash and the fluorescence monitored, to ascertain whether significant bleaching of the two dyes occurred.

(g) Length of flash artefact:

The PMT was protected from the UV flash by a barrier filter. It measured fluorescence continuously throughout the experiment, although a flash-artefact was recorded at the moment of the UV flash and persisted for a number of milliseconds. The length of the flash artefact was determined by flashing at an uninjected cell and measuring the time taken for the fluorescence to return to baseline values (see section 3.1.5). This was found to be no more than 100 ms. After-effects of a bright flash in the PMT are unlikely to persist for 100 ms, so the artefact is likely to be due to phosphorescence of some component of the optical system.

(h) Absorption of the UV flash across the cell:

Since the NP-EGTA introduced into the cell will absorb the UV flash, it is important to ascertain that this absorption is not significant. The equation $A=\varepsilon bc$ (where A is absorption, ε is the extinction co-efficient of the compound, b is the path length of light through the cell and c is the concentration) can be applied.

For a final concentration in the cell of 500μM NP-EGTA (higher than I normally used) and a cell diameter of 30μm;

$\varepsilon = 974\ M^{-1}cm^{-1}$ (Ellis-Davies and Kaplan, 1994)

$b = 0.003cm$

$c = 5 \times 10^{-4}M$

applying:

$A = \varepsilon bc$

$A = 1.461 \times 10^{-3}$

Since $A = \log_{10} \frac{I_o}{I}$

where I_o is intensity of light incident on the cell

and I is the intensity of light leaving the cell,

the amount of incident UV absorbed by the NP-EGTA introduced into the cell is 0.3%, a negligible fraction.

(i) Calcium Calibrations for the Buffering Experiments:

In vitro calibrations of the SR/F/NP injection solution were made. The full injection solution was used for each calibration so that any effect of the NP-EGTA on the fluorescence spectra of the dyes was allowed for. The reason for this precaution is that the caged calcium compounds DM-nitrophen and Nitr-5 have been found to affect the fluorescence spectrum of Fluo-3 by quenching the fluorescence of Fluo-3 under certain conditions, and by distortion of the spectra occurring through calcium-dependent fluorescence of the chelators themselves

(see section 1.1.4(b)) (Zucker, 1992). The absorption spectra of photolysed NP-EGTA, calcium-saturated and calcium free NP-EGTA do not differ with respect to one another and show that NP-EGTA is virtually non-fluorescent (Ellis-Davies and Kaplan, 1994). The excitation wavelengths used to measure the fluorescence of the sulforhodamine and Fluo-3 do not photolyse the NP-EGTA. Nevertheless, to eliminate any possibility of interference with calcium calibrations from the compound, all calibrations were performed using the full injection solution.

Ratios of Fluo-3 fluorescence to sulforhodamine fluorescence were made in zero calcium solution (in mM: KCl 140, EGTA 10, HEPES 10, pH 7.2) and in a solution of saturating calcium (in mM: KCl 140, $CaCl_2$ 10, HEPES 10, pH 7.2), to ascertain values for R_{min} and R_{max} respectively. These values were checked weekly since they are highly dependent on the output of the excitation light source. The values were used for all calcium calibrations applied to the cells.

The fluorescence measurements for the ratios were made with a neutral density filters totalling OD 1.4 present in the path of the excitation beam. This was to reduce the fluorescence intensity of principally the sulforhodamine element of the injection solution since at high concentrations of the dye, the photomultiplier output began to saturate. All ratios used in calcium calibrations in the cell were also made with the same neutral density filters present in the excitation light path, that is, the calculation of calcium does not assume that the neutral density filters were perfectly achromatic.

(j) Ryanodine experiments:

For the model for buffering being proposed, it is important that no calcium-induced-calcium-release (CICR) occurs in these cells. To ascertain whether CICR does occur, the amount of calcium released after an UV flash when the cells were loaded with >200μM NP-EGTA was compared in cells in the presence or absence of ryanodine. Cells were injected with the SR/F/NP mixture and the dish rinsed after injection with fresh HBS. Stock solution of ryanodine was added to the dish to a final concentration of 200μM and a minimum of ten minutes allowed before readings were taken. The same measurements as outlined above were made. Cells used in the two experimental protocols were from the same batch of cells and so differences due to batch-batch variability were eliminated. If CICR occurs in these cells, a greater rise in $[Ca^{2+}]_i$ would be expected in cells not treated with ryanodine.

(k) Buffering capacity of annexin VI$^+$ cells:

A comparison of the buffering capacity of wtA431 cells and A431 cells transfected with and expressing annexin VI was made. Both sets of cells were injected with the SR/F/NP injection solution as outlined above. A comparison of the rise in calcium seen after a single UV flash was made. Another group of cells were also loaded before injection with BAPTA AM (see section on AM loading - 2.1.3). These cells were then injected as described, and exposed to a single UV flash.

(l) Image Acquisition:

Cells loaded with Fura-2 were maintained in HBS and images for the calculation of the ratio measured. Pairs of images for analysis were taken continuously throughout the experiment at approximately 15 second intervals unless otherwise indicated.

Ratiometric measurements were made by measuring fluorescence emission through a 510 nm broadband filter (Glen Spectra) during alternate excitation at 350 nm and 380 nm (see section "The epifluorescence microscope" in section 2.1.5). Images were collected via the intensified CCD camera and stored on the hard drive of the computer. The intensity of emission fluorescence was measured for both a background region containing no cells and for each cell to be analysed using commercial image analysis software (Global Lab Image, Data Translation, England). Hence a background-corrected ratio of emission from 350 nm excitation to emission from 380 nm excitation could be calculated. From this, and an *in vitro* calcium calibration, the concentration of calcium within the cells could be calculated.

(m) Cross talk protocol:

In all experiments, wtA431 cells were AM loaded with Fura-2 as described above (section 2.1.3). The cells were then rinsed with HBS and mounted on the microscope. A suitable field of cells was found and pairs of images of the cells taken approximately every 15 seconds. Two pairs of images before stimulation were taken with the cells simply in HBS, and the cells were stimulated according to the following protocols, images being taken throughout. The time of addition of

agonist was set as t = 0, and so resting images are indicated by negative time values. Aliquots of stock solutions were made up to 10 mls of final concentration using HBS. 10 mls is sufficient for two dishes of cells and any surplus remaining after the two dishes was discarded. Hence no solution of agonist was at room temperature for longer than 40 minutes. No appreciable difference in response was observed, indicating that degradation of the agonist had not occurred. Stock aliquots remained in the freezer or on ice until required.

Problems associated with the timing of the drug applications:

The timing of drug applications of the two agonists was important. With the EGF response following prior stimulation with BK, the response to BK needed to have finished with respect to intracellular calcium concentrations before attempting to measure calcium changes in the cells following perfusion with EGF. However, the action of the kinases and phosphatases implicated in EGF-R modulation may have a short lasting effect. Indeed the kinase and phosphatase activities are unlikely to have the same kinetics, and the timing of the two agonist applications may well reflect this.

In the work by Hosoi, Kurihara and Ueha (1993), maximum phosphorylation of the EGF-R on Thr-655 was at two and a half minutes. In the experiments reported in this thesis, EGF application was always four minutes after the application of BK. There is still significant phosphorylation of the EGF-R at five minutes, according to Hosoi, Kurihara and Ueha (1993), and the effect of the phosphoprotein phosphatase action appears only after two and a half minutes. A difference in percentage EGF bound to the EGF-R following BK stimulation, between control and phosphoprotein phosphatase-inhibited cells was only evident

after five minutes in the work by Hosoi, Kurihara and Ueha (1993). Thus if the phenomena described by Hosoi *et al* operate to modulate EGF-R sensitivity, my protocol should detect it.

The BK calcium response is a sharp peak with no following plateau, and is purely from internal stores. Not only could there be interactions between BK and the EGF-R affecting the response, but, since EGF also releases calcium from internal $InsP_3$-sensitive stores, any effect of store depletion and/or potentiation of the $InsP_3$ system will have an effect. Again, the application of EGF following BK pre-treatment must be soon enough to allow for such (probably) short-term effects to remain, but long enough after BK stimulation to allow intracellular free calcium levels to return to baseline levels.

More problems were encountered when applying BK after pre-treatment of the cells with EGF. Low concentrations of the growth hormone have short-lasting calcium responses, but higher doses have calcium concentration changes in the cell that last for many minutes. Since a range of concentrations were used for pre-treatment, some standard period of time between agonist applications had to be adopted. The treatment sequence selected reflects the difficulty in choice between allowing the cell calcium levels to return towards normal following EGF stimulation with high doses, and maintaining a short enough interval between agonists to protect the interactions occurring in the cell signalling pathways.

EGF stimulation following BK stimulation

At t = 0, 1μM BK was perfused through the bath for 60 s. This was followed by 180 s of washout with HBS, then various concentrations of rhEGF, from

10ng/ml to 100ng/ml rhEGF were perfused through the bath. Cells were exposed to rhEGF for approximately five minutes.

BK stimulation following prior stimulation with rhEGF

At t = 0, various concentrations of rhEGF, from 10ng/ml to 100ng/ml were perfused through the bath. Cells were exposed to rhEGF for five minutes. This was followed by a 60 s washout with HBS, then 1μM BK was perfused through the bath and the cells monitored for a further 120 s.

(n) Protocol for experiments on the wild type and annexin transfected cells:

Loading of cells and calcium measurements:

Dishes of cells were AM loaded with Fura-2 as described above. Following loading, the cells were rinsed with HBS and a perfusion chamber attached to the coverslip (or base of the dish in the case of the Petriperms), with high vacuum grease. The cells were then mounted on the epifluorescence microscope and a suitable field of view found.

Protocol:

Cells were maintained in HBS and the resting ratio measured. Images for analysis were taken continuously throughout the experiment at approximately 15 second intervals unless otherwise indicated. Various solutions were applied to the cells as outlined below. Aliquots of stock solutions were made up to 10 mls of final concentration using the appropriate HBS solution. 10 mls is sufficient for two dishes of cells and any surplus remaining after the two dishes was discarded. Hence no solution of agonist was at room temperature for longer than 40 minutes.

No appreciable difference in response was observed, indicating that degradation of the agonist had not occurred. Stock aliquots remained in the freezer or on ice until required. At t = 0, either rhEGF (various concentrations) or bradykinin (BK) were introduced into the bath through the rapid perfusion system. Cells were monitored for approximately 5 mins. In experiments with rhEGF in calcium free saline, calcium free HBS was run into the perfusion bath for a minimum of 30 s before perfusion with rhEGF in calcium free saline. The time that agonists were applied to the cell is given as t = 0, and the resting images are therefore considered as being taken at negative times.

(o) Neomycin:

All the transfected cells (both controls and annexin VI^{+} clones) were cultured in media containing neomycin. Neomycin kills any cells that revert to wild type and therefore fail to express the neomycin resistance gene. Neomycin acts by inhibiting the mRNA-ribosome-tRNA complex. However, the selectivity of its action is not absolute It is recognised as an inhibitor of phospholipase C and D (McDonald and Mamrack, 1995; Golding and Vink, 1994). Thus the residual amount of neomycin not inactivated by the product of the resistance gene might be compromising the response of the transfected cells to rhEGF. I therefore tested the response of one of the control transfectants to rhEGF following removal from the presence of neomycin for 7 days prior to the experiment. The same protocol for rhEGF stimulation as is outlined above was followed.

(p) Manganese quench:

The Till monochromator allowed me to determine the effective iso-fluorescent wavelength by setting the excitation to each of a series of wavelengths and stimulating Fura-2 loaded cells with BK. The iso-fluorescent wavelength was determined to be 360 nm. This wavelength was used to monitor the amount of bleaching that occurs when manganese enters the cell and binds to Fura-2. Cells were prepared and Fura-2 AM loaded as already described. Cells were rinsed with and maintained in HBS and mounted on the Till epifluorescence microscope. The intensity of fluorescence emission at 510 nm following excitation at 360 nm of the Fura-2 was measured every 15 seconds. After three minutes, either HBS supplemented with 0.5mM $MnCl_2$ (Mn-HBS) or Mn-HBS further supplemented with 100ng/ml rhEGF was perfused through the bath and fluorescence emission intensity monitored for approximately five minutes. The data was normalised to the initial fluorescence emission. Following normalisation the mean and SEM were calculated for each cell type at each time point.

(q) Calcium calibrations for the experiments on calcium signalling in wtA431 cells and transfected cells:

Ratios obtained from analysis of the images taken throughout the experiment were converted to calcium concentrations by comparison with an *in vitro* calibration. Solutions of the free acid form of Fura-2 in zero calcium solution (in mM: KCl 140, EGTA 10, HEPES 10, pH 7.2) and saturating calcium solution (in mM: KCl 140, $CaCl_2$ 10, HEPES 10, pH 7.2), were prepared, loaded into microcuvettes (Camlab, Cambridge, England) (path length 50μm) and the ratios

of emission fluorescence at 510 nm following excitation with 350 nm and 380 nm light calculated. These ratios are referred to as R_{min} and R_{max} respectively.

Viscosity Correction:

Poenie (1990) found that Fura-2 was affected by the viscosity of its environment. Changes of viscosity altered the relative amplitude of emission during excitation at 340, 350 and 380 nm, Ca:free and Ca:bound forms of Fura-2 being affected to the same extent. K_d is unaffected. Thus, intracellular calcium ($[Ca^{2+}]_i$) can be calculated as follows.

$$[Ca^{2+}]_i = Kd * \frac{Sf,\lambda 2}{Sb,\lambda 2} \frac{R/V - R_{min}}{R_{max} - R/V}$$

where

Kd = calcium concentration when half the dye is saturated with calcium

R = R value recorded from intracellular Fura-2

R_{min} = R value recorded from an *in vitro* solution at zero calcium

R_{max} = R value recorded from an *in vitro* solution saturated with calcium

V = the correction factor to compensate the difference in viscosity between the *in vitro* and intracellular environments

$Sf,\lambda 2$ = fluorescence from 380 excitation of calcium free solution *in vitro*

$Sb,\lambda 2$ = fluorescence from 380 excitation of solution saturated with calcium *in vitro*

R_{min}, R_{max}, Sf,λ2 and Sb,λ2 are parameters which are unique to each imaging system and depend on the light source and optical system used.

Direct measurement of V (Poenie, 1990)

This method involves introducing a $[Ca^{2+}]_i$ change in the cytosol in conditions where the intracellular dye concentration is not altered. Knowledge of the value of the calcium change is not necessary. The fluorescence at each excitation wavelength will change, by $\Delta I_{\lambda 1}$ and $\Delta I_{\lambda 2}$, respectively. Dividing $\Delta I_{\lambda 1}$ by $\Delta I_{\lambda 2}$, each measured in the intracellular environment, gives the ratio Z_i. This process is repeated *in vitro*, using the same conditions in which the R_{min} and R_{max} were measured. The ratio $\Delta I_{\lambda 1}/\Delta I_{\lambda 2}$, measured in the *in vitro* environment is called Z_e. V (viscosity correction factor) is given by Z_i/Z_e.

Viscosity Correction for A431 cells

Poenie's method of determining the correction for the effect of viscosity was followed. Z_i was calculated from experiments on wtA431 cells that were stimulated with rhEGF at 100ng/ml, and is an average of Z_i for each wtA431 cell which showed a large calcium change (i.e. cells in which the ratio after stimulation was greater than 1 or cells in which the resting calcium was high and fell dramatically). The measurements were repeated for A6.1 cells to check that transfection and expression of the annexin VI protein would not significantly affect the viscosity correction calculations. The results were as follows:

A431 cells		A6.1 cells	
mean Z_i	-0.207	mean Z_i	-0.295
stdev	0.252	stdev	0.31
N	56	N	32
SEM	0.034	SEM	0.055

An unpaired T-test was applied to the results and indicated that there is no significant difference between Z_i in the two cell types ($p > 0.05$).

The *in vitro* value Z_e was calculated as follows. A minimum of six separate dilutions for each concentration point, i.e. saturating calcium and zero calcium were performed. The intensifier gain was set to be below saturation for excitation with 380 nm light with the calcium free sample, since this was the brightest of all the conditions, and then turned down further to allow leeway for the other microcuvettes. The gain was not altered for the whole of the experiment. Microcuvettes were filled with solution and the surface of the microcuvette focussed on. The two calcium concentration series were worked through in the order: calcium-free (350, 380), background (350, 380), saturating calcium (350, 380), background (350, 380); where each background was a microcuvette of the calibration solution with no dye present and the figures represent the excitation wavelength.

The data was analysed to produce a ratio for each dilution of each concentration and the mean of the results found. The mean 380 nm fluorescence intensities for both calcium free and saturating calcium were found, to calculate the Sb,λ2 and Sf,λ2. Z_e was calculated as outlined above.

From the calibration, the following results were obtained:

$R_{min} = 0.313 \pm 0.014$, $R_{max} = 16.283 \pm 0.372$

$Z_e = -0.529 \pm 0.093$

V, the viscosity factor is calculated from Z_i / Z_e. Using the A431 data, and the *in vitro* calibration data, the value of V is:

$Z_i =$ -0.207

$Z_e =$ -0.529

$V =$ 0.3917

This viscosity correction factor was used when converting from ratios to calcium concentrations in all the experiments with parental and transfected A431 cells.

BUFFERING CAPACITY

3.1 PRELIMINARY WORK

3.1.1 Determination of resting calcium levels in the cells

It was important to be able to measure the calcium concentration within the cell, to be able to ascertain both the resting calcium level of the cell and the change in calcium caused by release of calcium from the caged compound introduced into the cell. Since the method commonly used to accurately measure calcium concentrations involves the use of ratios to nullify the effects of dye concentration in the cell, and the best characterised ratiometric calcium indicators use ultraviolet light for the excitation wavelength, it was necessary to design a visible wavelength ratiometric dye system. Excitation with UV light would destroy the photolabile calcium chelator being used to release calcium into the cell. A combination of the calcium-sensitive dye Fluo-3 (F) with a calcium-insensitive dye, sulforhodamine 101 (SR) was used. The ratio of Fluo-3 fluorescence to SR fluorescence with neutral density filters of 1.4 present in the excitation beam was measured at the start of each experiment. This ratio was compared with an *in vitro* calibration and the $[Ca^{2+}]_i$ in the cells calculated according to the equation:

$$[Ca^{2+}]_i = Kd * \frac{(R-R_{min})}{(R_{max}-R)}$$

where Kd is the calcium concentration at which Fluo-3 is 50% bound with calcium,

R_{min} is the ratio when the calcium concentration is zero and all the Fluo-3 is unbound,

and R_{max} is the ratio when the calcium concentration is at saturating levels and the Fluo-3 is all in the calcium bound form.

The mean resting value for $[Ca^{2+}]_i$ was found to be 45.4nM. Any cells with a resting $[Ca^{2+}]_i$ of >100nM were rejected from further analysis. 100nM is an arbitrary cut-off point, but since the majority of the cells had a resting calcium concentration of far less than this and cells that had a resting calcium concentration of much greater than 100nM generally appeared damaged or sick, this concentration was used as a set cut-off point.

3.1.2 Calcium calibration and value of K

Two different injection solutions were used over the course of the experiments. The values of R_{min}, R_{max} and K, a constant used in the determination of SR concentration within the cell (see section 2.2.1), were ascertained regularly for each solution and did not vary significantly from the following values:

Injection solution 1:		Injection solution 2:	
R_{min} =	0.001244	R_{min} =	0.003998
R_{max} =	0.069704	R_{max} =	0.435295
K =	1.23×10^{15} cps m^{-3} M^{-3}	K =	4.38×10^{14} cps m^{-3} M^{-3}

Differences between the values are assumed to be the result of different batches of constituent compounds being used to produce the injection solutions. The appropriate calibrations were applied to cells for analysis.

3.1.3 Cell damage causing a rise in calcium

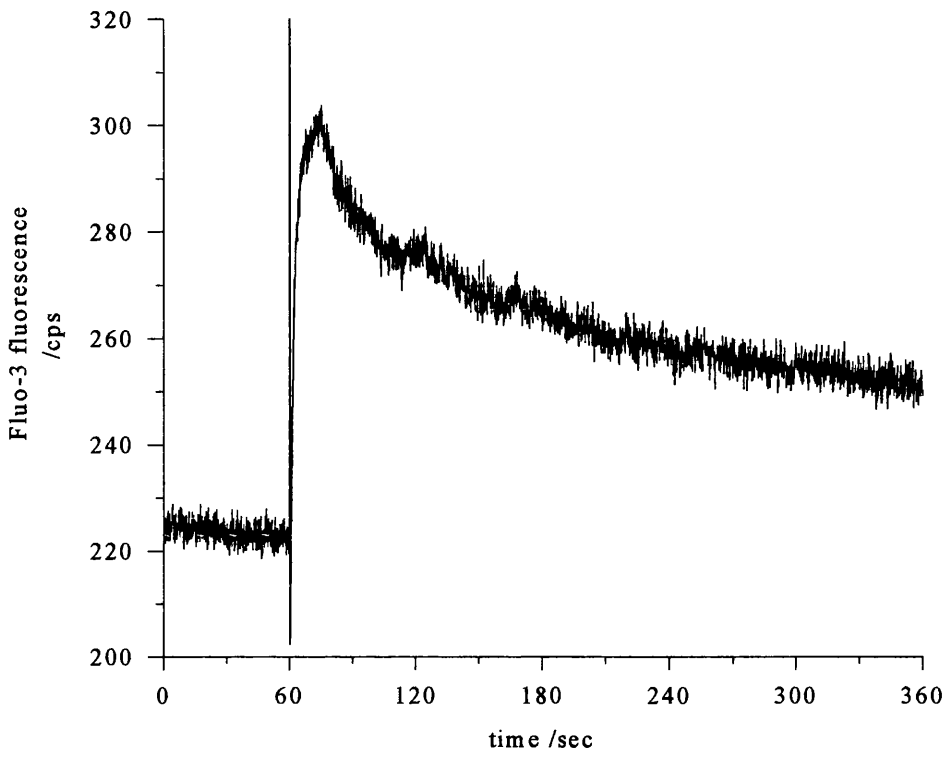
In preliminary work, it was noticed that there was a rise in calcium in the cells after exposure to an UV flash, even if the cells had not been injected with any caged calcium compounds (see figure 3.1.3a). Since the process of injecting the cells is by its very nature damaging to the cells, a second set of cells were AM loaded with Fluo-3 AM and subjected to a single UV flash. The results of this are shown in figure 3.1.3b. As can be seen, the post-flash rise in calcium persists.

Cultured cells are also thought to be more fragile than primary cells. Dorsal root ganglion cells were loaded with Fluo-3 AM and exposed to a single UV flash (figure 3.1.3c). The cells still displayed a rise in calcium that was unrelated to any release from a caged compound being present in the cell.

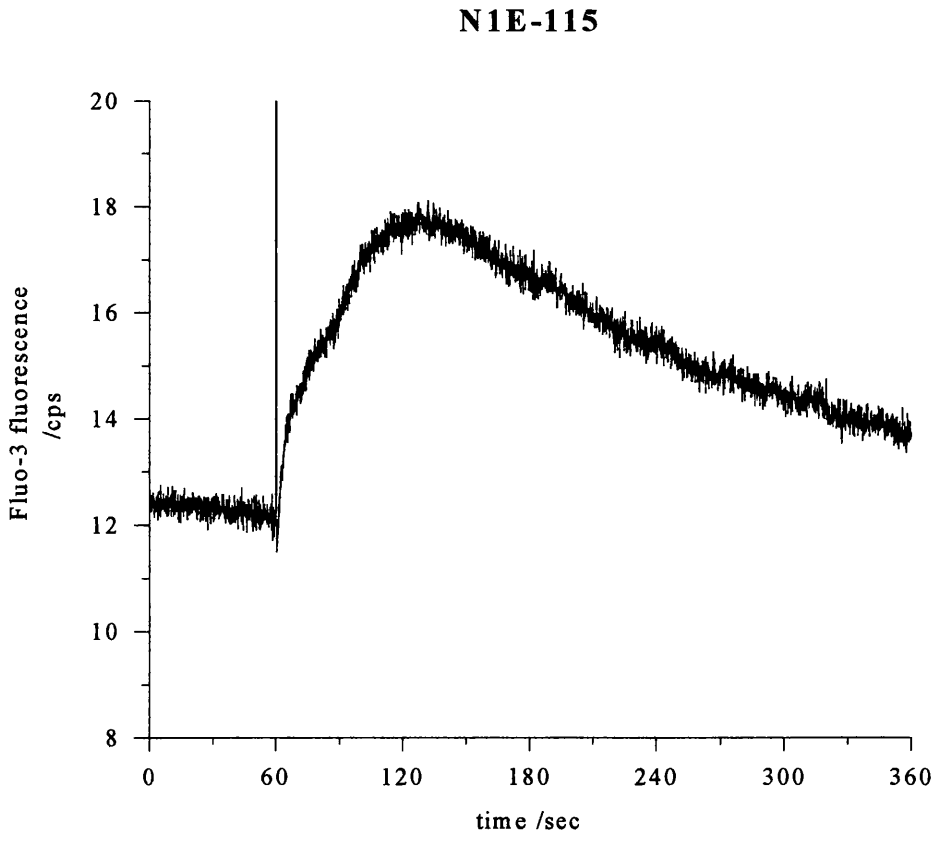
Since the high energy UV flash could be producing free radicals in the cell, the experiment was repeated in the presence of the free radical scavenger, 4-OH TEMPO (figure 3.1.3d) and the anti-peroxidase enzyme catalase (figure 3.1.3e) but to no avail. Finally, the UV flash was attenuated by inserting a 0.6 optical density filter into the path of the UV flash. N1E-115 cells were AM loaded with Fluo-3 and exposed to a single UV flash (figure 3.1.3f). It can be seen that the cell (representative of four cells) displayed no rise in calcium from cell damage and hence, this optical density filter was routinely included in the light path.

Figure 3.1.3:

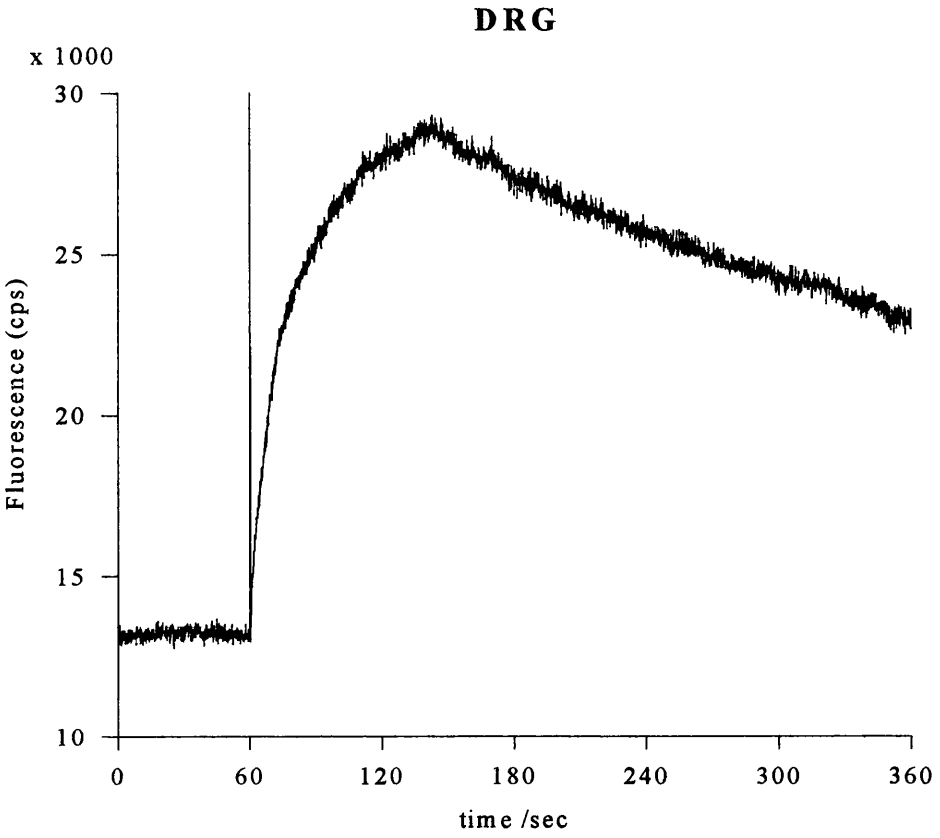
(a)



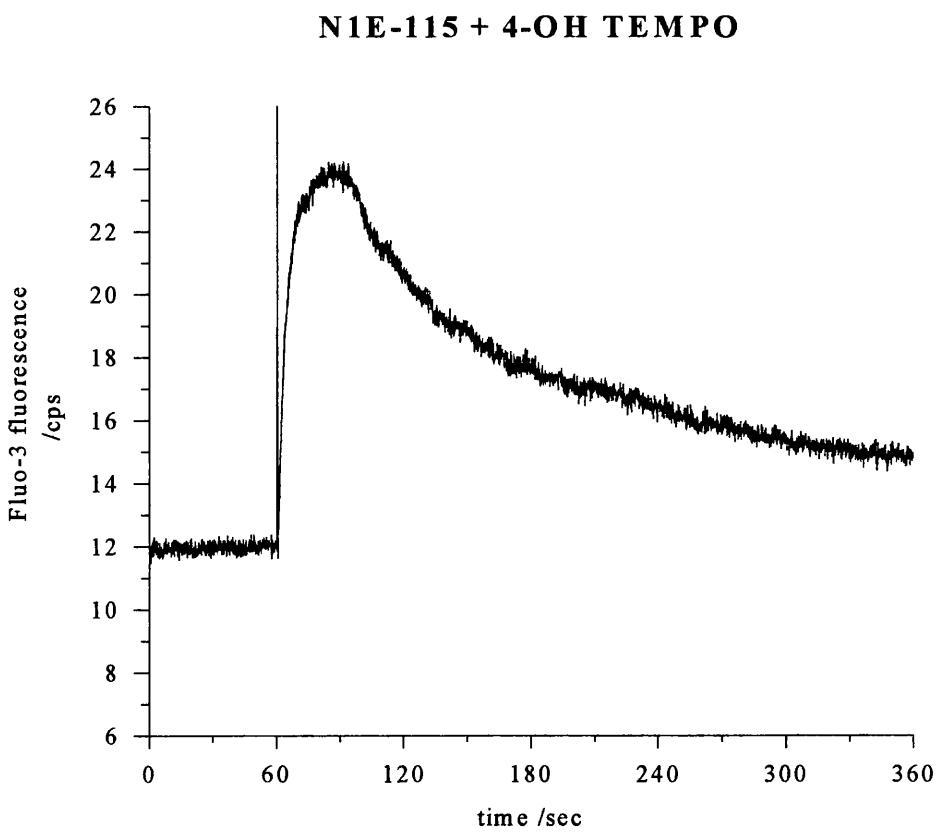
(b)



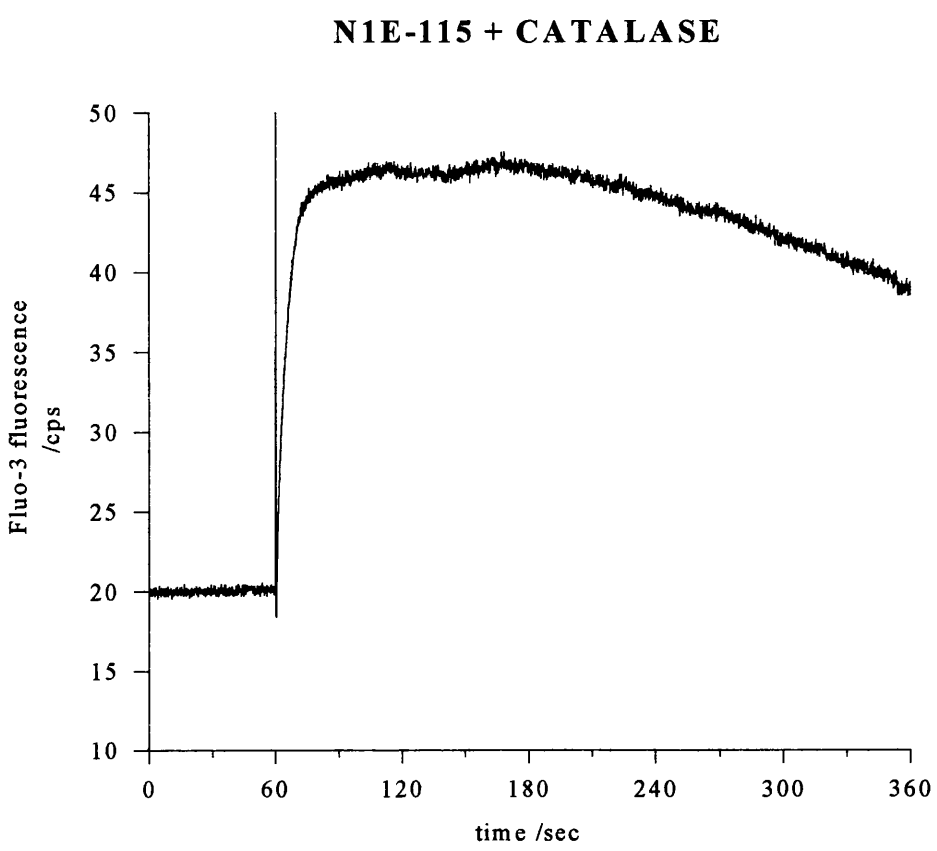
(c)


DRG
x 1000
30
25
20
15
10
Fluorescence (cps)
0
60
120
180
240
300
360
time /sec

(d)


N1E-115 + 4-OH TEMPO
26
24
22
20
18
16
14
12
10
8
6
Fluo-3 fluorescence /cps
0
60
120
180
240
300
360
time /sec

(e)


N1E-115 + CATALASE
Fluo-3 fluorescence /cps
50
45
40
35
30
25
20
15
10
0
60
120
180
240
300
360
time /sec

(f)

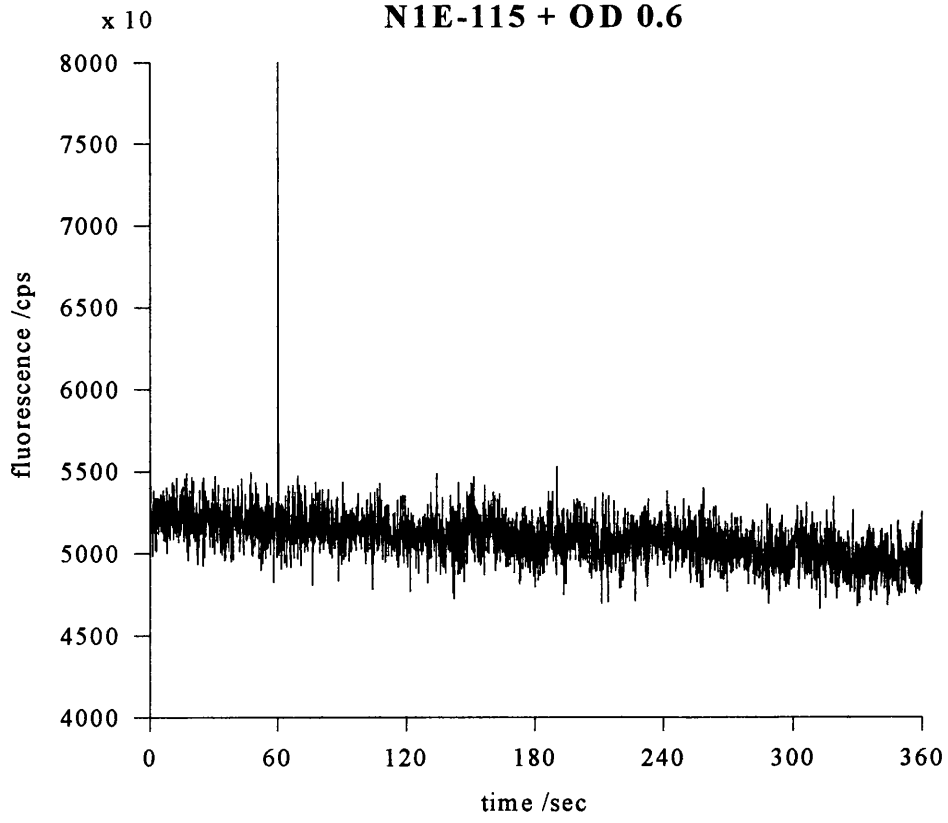

N1E-115 + OD 0.6
x 10
fluorescence /cps
8000
7500
7000
6500
6000
5500
5000
4500
4000
0
60
120
180
240
300
360
time /sec

Figure 3.1.3: Cell damage.

In all cases, the trace is a representative trace of several experiments.

(a) N1E-115 cell injected with Fluo-3 alone - there is no concomitant injection of NP-EGTA.

Following the UV flash (indicated by the artefact - at approximately t = 60 s) there is a rise in Fluo-3 fluorescence that peaks, then plateaus at a higher level than the resting state.

(b) N1E-115 cell that has been AM loaded with Fluo-3.

Following the flash there is still a rise in fluorescence, indicating that cell damage from injection of the cells has not compromised the cells, leading to a rise in $[Ca^{2+}]i$ on further assault.

(c) Dorsal root ganglion cell, AM loaded with Fluo-3 AM.

This also shows the phenomenon of increased $[Ca^{2+}]i$ following the UV flash, indicating that it is not a problem related to cultured cells.

(d) N1E-115 cell + 4-OH TEMPO (a free radical scavenger).

Inclusion of 500μM 4-OH TEMPO in the saline bathing the cell does not attenuate the post-flash calcium rise. Cell was AM loaded with Fluo-3 AM.

(e) DRG cell + catalase (a free radical scavenger).

Inclusion of 125u/ml catalase in the saline bathing the cells does not attenuate the post-flash in calcium. Cell was AM loaded with Fluo-3 AM.

(f) N1E-115 cell that was loaded with Fluo-3 AM. The UV flash has been attenuated by insertion of a 0.6 optical density filter in the optical path of the flash gun. The post-flash rise in calcium has been totally abolished by this inclusion, indicating that the unattenuated flash was causing damage to the cell, possibly at the cell membrane, leading to increased $[Ca^{2+}]i$.

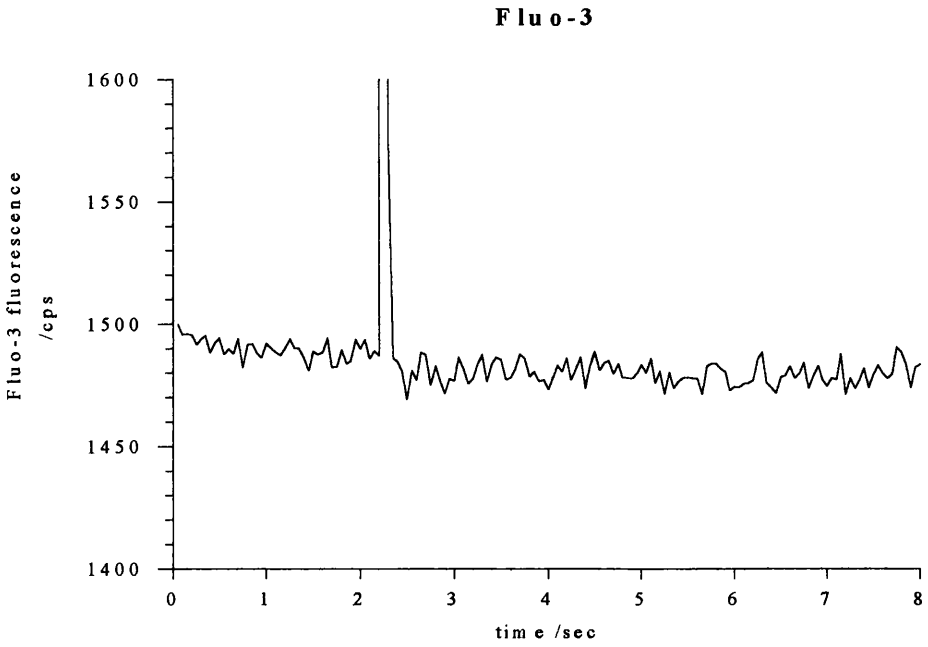
3.1.4 Photobleaching of Fluo-3 and Sulforhodamine 101

It was important that neither of the dyes used to calculate the calcium concentration within the cell were bleached by the UV flash. A solution of 10μM Fluo-3 (free acid) or 10μM sulforhodamine 101, in 20μM $CaCl_2$, 150mM HEPES was loaded into a microcuvette and exposed to a single UV flash (figure 3.1.4 a, b). The solution of Fluo-3 was bleached by approximately 0.8% and the sulforhodamine 101 not at all. It should be noted that the flash that the microcuvettes were exposed to was not attenuated by the optical density filter. In all the experiments reported below the UV flash was attenuated by a 0.6 OD filter; thus in the experiments that follow, bleaching of Fluo-3 will have been insignificant.

3.1.5 The flash artefact

The photomultiplier tube was shielded from the UV flash by the optics of the system, which allowed only light of wavelength 300 to 450 nm through and hence protected the PMT from damage, without the use of a shutter. However, a flash artefact remained in the recordings. The length of the flash artefact was determined by exposing an uninjected cell to a single UV flash and measuring the time taken for the PMT counts to fall back to pre-flash levels. The result of one such experiment is shown in figure 3.1.5 (representative of three experiments). The artefact is judged to be over by 100 ms.

(a)



(b)

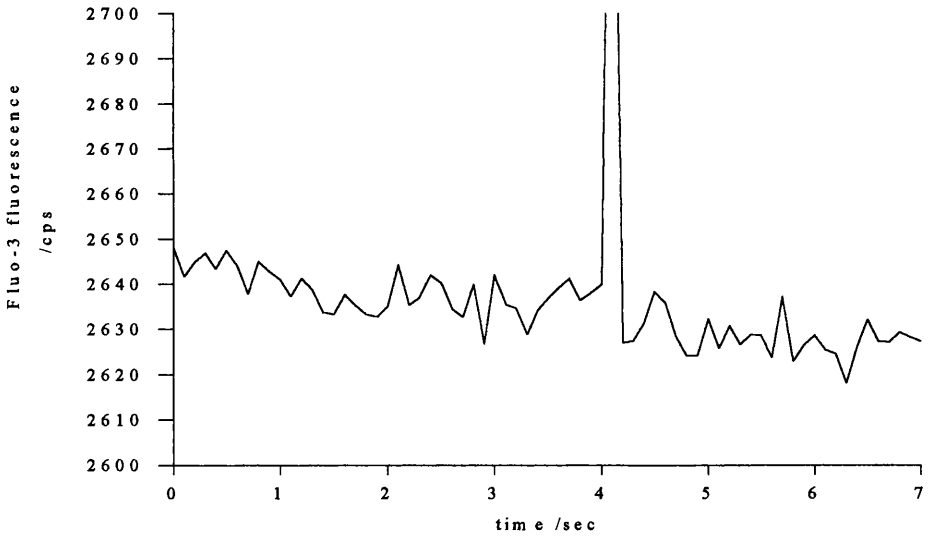


Figure 3.1.4: Photobleaching of Fluo-3 and SR.

(a) 10μM Fluo-3 (free acid) + 20μM $CaCl_2$ in 150mM HEPES, was exposed to a single flash from the UV gun. The solution was photobleached by approximately 0.8%.The flash was not attenuated by the inclusion of a 0.6 optical density filter in the optical path.

(b) A 10μM solution of sulforhodamine 101 was exposed to a single flash from the UV gun. No photobleaching was observed.

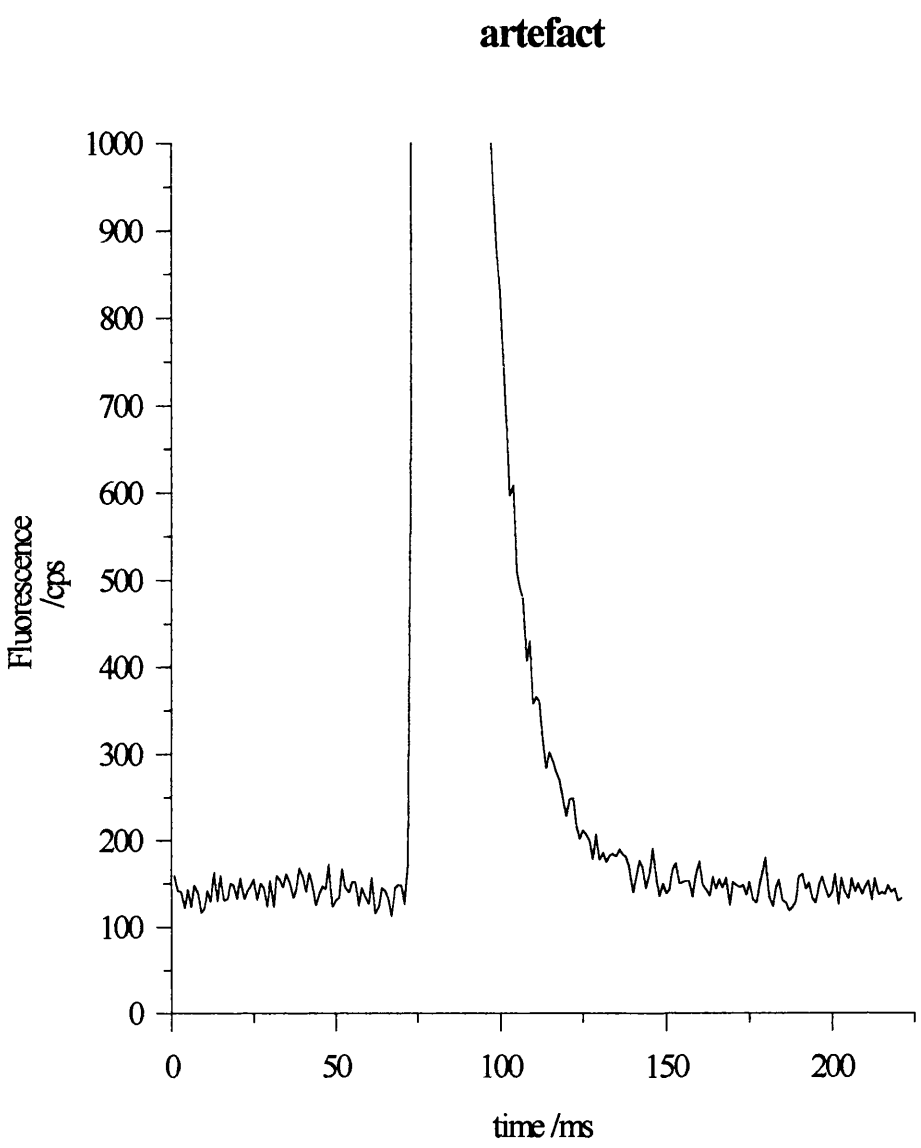


Figure 3.1.5: Flash artefact.

An uninjected cell was exposed to a single flash from the UV flash gun to determine the length of the flash artefact observed. The flash artefact is judged to persist for no longer than 100ms.

3.1.6 Comparison of Ca^{2+} changes in the presence or absence of ryanodine

Since the determination of the buffering capacity of the cell relies on knowing the total calcium added to the intracellular solution (as the release of calcium from the photolabile chelator), it was important that the small amount of calcium being released from the chelator was not triggering the release of intracellular calcium from stores via calcium-induced-calcium-release (CICR). The compound ryanodine blocks CICR by locking the calcium channels in the stores in either the open or closed state. To ascertain whether there was a component of the calcium change observed that was due to CICR, the experiment was repeated in the presence of 200μM ryanodine.

The mean ΔCa^{2+} from cells injected with >200μM NP-EGTA is 4.31± 1.05nM (n=8) in the presence of 200μM ryanodine, and 3.45± 0.82nM (n=12) in the absence of ryanodine. These values are not significantly different according to a t-test ($p > 0.05$). Ryanodine was not used routinely in the experiments thereafter.

3.2 DETERMINATION OF BUFFERING CAPACITY

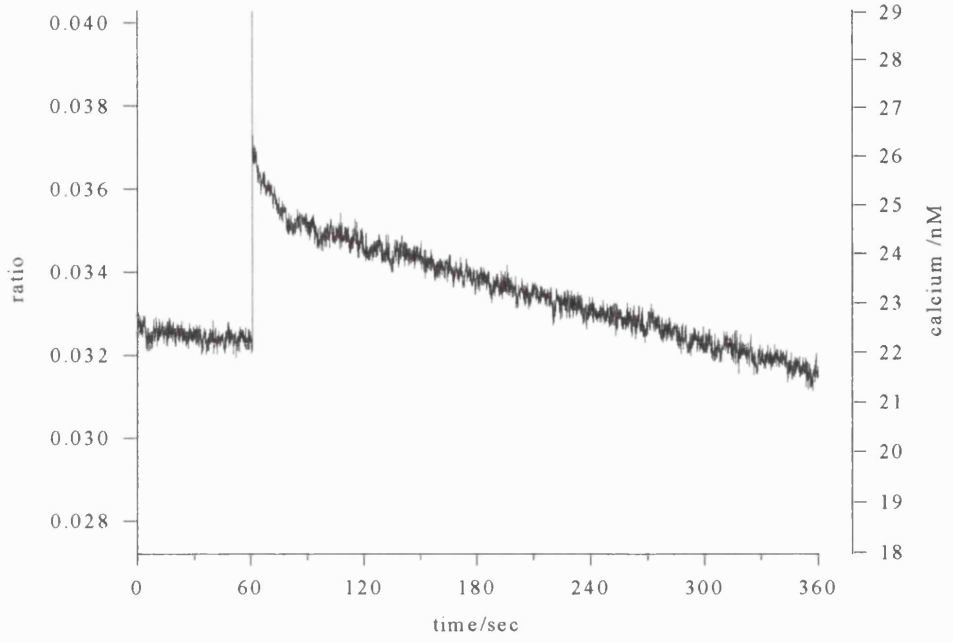
3.2.1 Protocol and results

An N1E-115 cell was injected with the injection solution, as described earlier. At the start of the experiment, readings were taken of the resting Fluo-3 and sulforhodamine fluorescence in the presence of the 1.4 OD filter in order to calculate the resting calcium concentration. Appropriate backgrounds were also taken. For the duration of the rest of the experiment, the OD filter was removed from the excitation light path to allow for greater resolution of calcium changes, and only Fluo-3 fluorescence was monitored. At approximately t = 60 s, the cell was exposed to a single UV flash and monitored for a further five minutes. The optical filter was then replaced and a second set of measurements for calculating the ratio and hence calcium concentration were made. Any fall in the sulforhodamine signal between the start and finish of the experiment could then be accounted for and incorporated into the calculations. A typical result is shown in figure 3.2.1 a, b. In the calculation of the calcium change, the flash artefact was removed from the trace and an exponential fitted to the data to allow extrapolation back to the moment of the flash. From this, the calcium before and after the flash could be calculated and therefore the change in calcium produced by the destruction of the NP-EGTA could be calculated.

The dimensions of each cell was measured prior to the experiment as described earlier (section 2.2.1a) and the volume calculated. From this, and the SR fluorescence values, the concentration of each of the components of the injection solution could be calculated, since there is a constant relationship between the concentrations. The fluorescence of the sulforhodamine is

determined solely by its concentration, since its fluorescence is not sensitive to any of the constituents of the cell. The volume of each cell was calculated as described and since concentration and fluorescence are linked by a simple equation (concentration = K * fluorescence * volume) and the constant K had been determined experimentally, the concentration of sulforhodamine could be calculated. Since the resting calcium of each cell is known, and the concentration of each component of the injection solution introduced to the cell was known, the concentration of Ca:NP-EGTA can be calculated. From this data, a plot of calcium change against Ca:NP-EGTA could be produced.

(a)



(b)

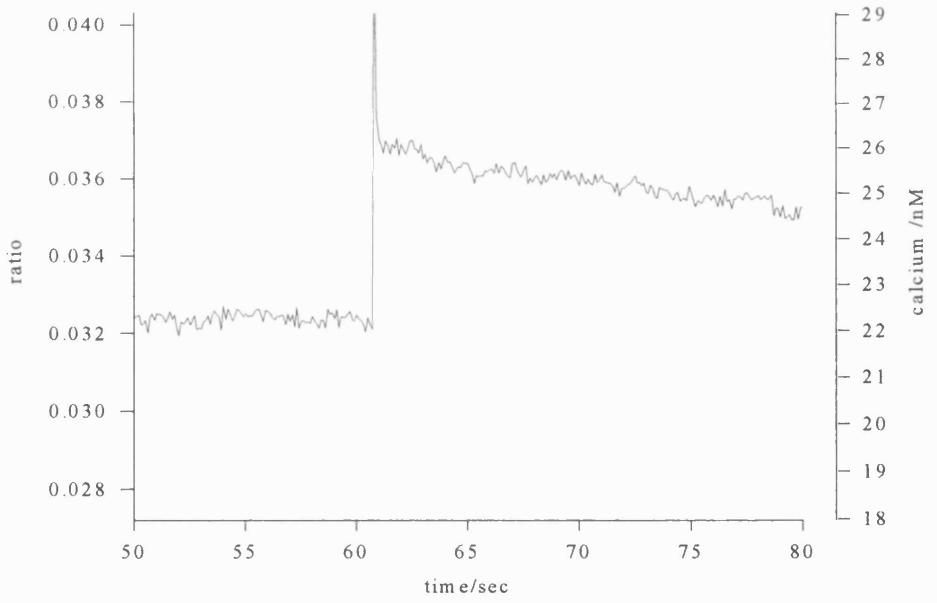


Figure 3.2.1: Example of a result.

(a) Typical result from N1E-115 cell injected with NP-EGTA, Fluo-3 and sulforhodamine 101. The flash occurred at approximately t = 60 s, causing an increase in $[Ca^{2+}]i$. This increase falls back to baseline over the duration of the experiment, approximately 5 mins.

(b) Same cell, on an expanded time scale.

3.3 CALCULATION OF BUFFERING CAPACITY

3.3.1 The proposed model

The relationship between the change in calcium observed for a given value of calcium-loaded NP-EGTA (Ca:NP-EGTA) was examined. This is shown in figure 3.3.1 a, b where the vertical axis is the change in calcium induced by the UV flash and the horizontal axis is the concentration of Ca:NP-EGTA in the cell. This is calculated from knowledge of the resting calcium concentration in the cell before the flash and the concentration of NP-EGTA that has been injected. In the lower range, where Ca:NP-EGTA is low, the change in calcium appears to show a steep dependence on Ca:NP-EGTA concentration (the relationship must pass through the origin). At higher concentrations of Ca:NP-EGTA, the increase in free calcium plateaus. I hypothesise that at low injection volumes, i.e., in the portion of the graph where the relationship is steep, the endogenous buffer of the cell is predominant. Under these conditions, an increase in the concentration of pre-flash Ca:NP-EGTA (that is, moving to the right along the graph) means that more Ca^{2+} is released during the flash to titrate the endogenous buffers - so the flash-induced calcium change is larger. Thus, for small Ca:NP-EGTA, ΔCa^{2+} will increase linearly with [Ca:NP-EGTA]. At higher injection volumes, the added buffer becomes predominant. Calcium released from photolysed NP-EGTA simply re-distributes onto the remaining, unphotolysed NP-EGTA and the Fluo-3 present in the cell. An increase in the concentration of pre-flash Ca:NP-EGTA (that is, moving to the right along the graph) means that more Ca^{2+} is released during the

flash, but also means that the amount of exogenous buffer has increased to match. Thus the flash-induced ΔCa^{2+} does not increase. Therefore, the graph levels off.

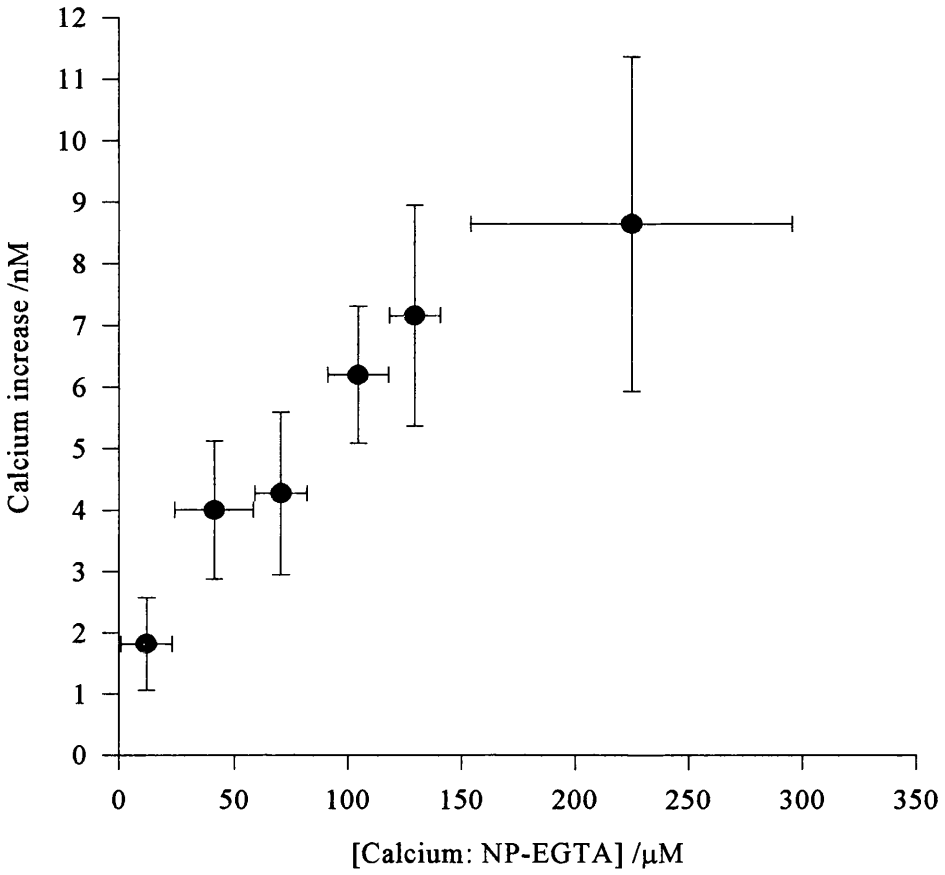


Figure 3.3.1: Plot of the results.

(a) A plot of $\Delta[Ca^{2+}]$ against Ca:NP-EGTA for N1E-115 cells. The vertical axis indicates the average change in $[Ca^{2+}]i$ following an UV flash; the horizontal axis denotes the concentration of Ca:NP-EGTA within the cell. Ca:NP-EGTA has been calculated from the concentration of NP-EGTA introduced into the cell and the resting $[Ca^{2+}]i$ before the flash. The data have been grouped and the horizontal error bars indicate the range of Ca:NP-EGTA of the group; the vertical error bars indicate the SEM of the $\Delta[Ca^{2+}]i$.

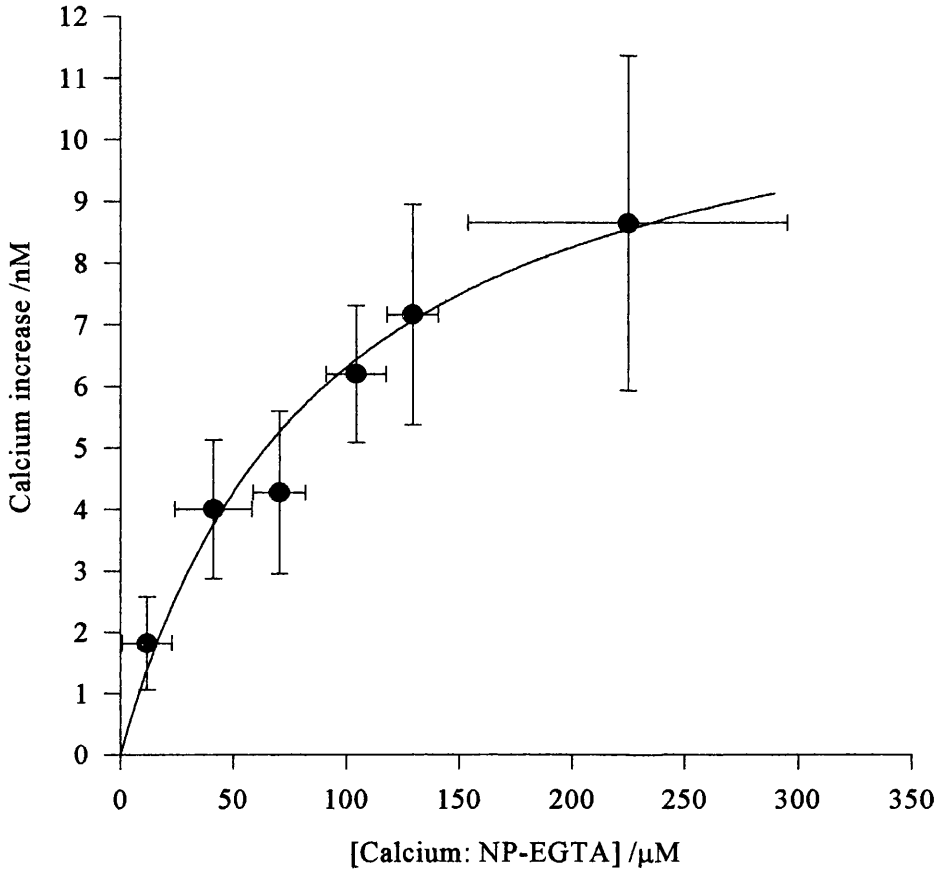


Figure 3.3.1 (b): A plot of Δ[Ca2+] against Ca:NP-EGTA for N1E-115 cells.

The same data from part (a) have been fit with an equation, where F = 22% +/- 8% and B = 1750 +/- 1250 (see text for details). At low concentrations of Ca:NP-EGTA, the endogenous buffers of the cell predominate. A small injection volume (and therefore low Ca:NP-EGTA concentration) produces a small increase in intracellular free calcium following destruction of the NP-EGTA by UV light. Hence the initial portion of the graph is linear. As injection volumes increase, the exogenous buffers introduced into the cell increase and eventually predominate and the graph plateaus.

To avoid making assumptions about the affinity of the cell's endogenous calcium buffers and to enable all endogenous buffers to be treated as a single entity, the buffering capacity of the cell will be described simply in terms of the number of calcium ions bound to cell constituents for every one that remains free in the cytosol.

The calcium released from the NP-EGTA is distributed within four pools. It can bind to the remaining, unphotolysed NP-EGTA in the cell, it can bind to Fluo-3 within the cell, it can bind to cell constituents - the endogenous buffering capacity of the cell, or it can remain free.

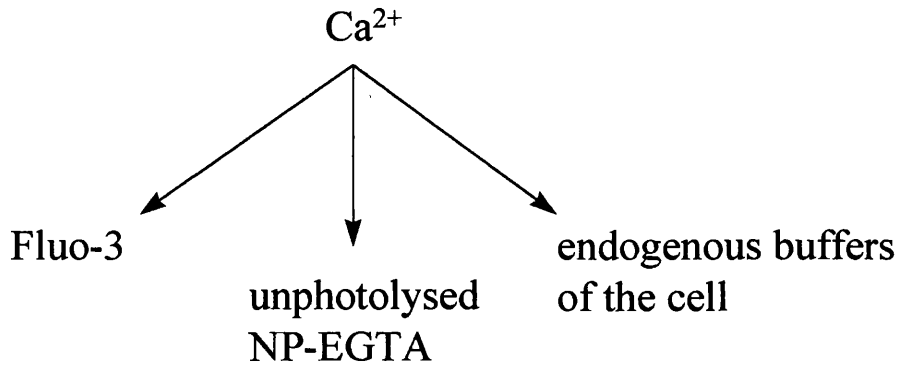


Figure 3.3.1: Distribution of calcium between four pools in the cell.

This can be expressed as follows:

I $[calcium_{TOTAL}] = [Ca^{2+}_{free}] + B*[Ca^{2+}_{free}] + [Ca:Fluo] + [Ca:NP\text{-}EGTA]$

where the endogenous buffering of the cell is described by the term B.

This expression can be differentiated to get:

$$\text{II}\quad \frac{d[\text{calcium}_{TOTAL}]}{d[Ca^{2+}\text{ free}]} = 1 + B + \frac{d[\text{Ca:Fluo}]}{d[Ca^{2+}\text{ free}]} + \frac{d[\text{Ca:NP-EGTA}]}{d[Ca^{2+}\text{ free}]}$$

Converting infinitesimals into real small changes and re-arranging, gives an expression for the change in free calcium resulting from a small change in total calcium:

$$\text{III}\quad \Delta[Ca^{2+}\text{ free}] = \frac{\Delta[\text{calcium}_{TOTAL}]}{1 + B + \frac{d[\text{Ca:Fluo}]}{d[Ca^{2+}\text{ free}]} + \frac{d[\text{Ca:NP-EGTA}]}{d[Ca^{2+}\text{ free}]}}$$

Expanding this equation fully, gives the expression below. There are two unknowns; B - the endogenous buffering of the cell and F - the percentage of NP-EGTA that is destroyed in a single UV flash. The term F appears twice in the equation; to calculate the amount of Ca^{2+} released from the NP-EGTA and also to calculate the amount of NP-EGTA remaining after the flash, since this is still a calcium buffer. Every other term in the equation is known.

$$\text{IV}\quad \Delta[Ca^{2+}\text{ free}] = \frac{F * [\text{Ca:NP-EGTA}]^{\text{pre-flash}}}{1 + B + \frac{Kd^{Fluo}[\text{Fluo}_{TOTAL}]}{(Kd^{Fluo} + [Ca^{2+}\text{ free}])^2} + \frac{Kd^{NP\text{-}EGTA}(1\text{-}F)[\text{NP-EGTA}]^{\text{pre-flash}}}{(Kd^{NP\text{-}EGTA} + [Ca^{2+}\text{free}])^2}}$$

The terms for Fluo-3 and NP-EGTA on the bottom line are derived by differentiating the equation

$$\text{V}\quad [\text{Ca:buffer}] = \frac{[Ca^{2+}\text{ free}][\text{buffer}_{TOTAL}]}{Kd + [Ca^{2+}\text{ free}]}$$

to give $\frac{d[Ca:buffer]}{d[Ca^{2+}\ free]} = \frac{Kd[buffer_{TOTAL}]}{(Kd + [Ca^{2+}\ free])^2}$

Sigmaplot was used to calculate those values of F and B that gave the best fit to the experimental data. The best fit values gave F (the percentage of NP-EGTA destroyed in a single flash) as 22% ± 8% and B (the buffering capacity of the cell) as 1707 calcium ions bound for every one calcium ion free ± 1455 (5% confidence limits) (figure 3.3.1b).

Another way of stating the buffering capacity would be to describe it as a single entity with a Kd for calcium and a concentration. If one assumes the Kd of the "buffer" is the same as the resting concentration of calcium in the cell, i.e., 45nM then the following can be derived from equation V above:

$$1700 = \frac{45*[buffer_{TOTAL}]}{(45 + 45)^2} = \frac{[buffer_{TOTAL}]}{180}$$

therefore $[buffer_{TOTAL}] = 306{,}000\ nM$

$= 306\mu M$

3.4 ANNEXIN VI

3.4.1 Is Annexin VI acting as a calcium buffer?

Since the annexin family of proteins are known to bind to phospholipids in a calcium dependent manner, I wondered whether the smaller increase in calcium concentration observed in the annexin transfected cells following stimulation with growth factors (see section 5.2.1), was due to the calcium binding to the annexin VI present in these cells.

Wild type A431 (wtA431) cells, wtA431 transfected with annexin VI and a further set of wtA431, loaded with BAPTA AM, were tested for their calcium buffering capacity, as outlined above.

If the mean change in calcium observed in the cells, following injection with between 200 and 500μM NP-EGTA is compared between the cell types (figure 3.4.1), it can be seen that there is no significant difference between the wild type cells and the transfected cells. The cells that had been loaded with BAPTA AM showed almost no increase in calcium following destruction of the NP-EGTA. It is assumed that the calcium released from the NP-EGTA is chelated by the BAPTA present in the cells. Since there is no difference observed between the transfected cells and the wild type, the annexin VI would appear not to be acting as a significant calcium buffer.

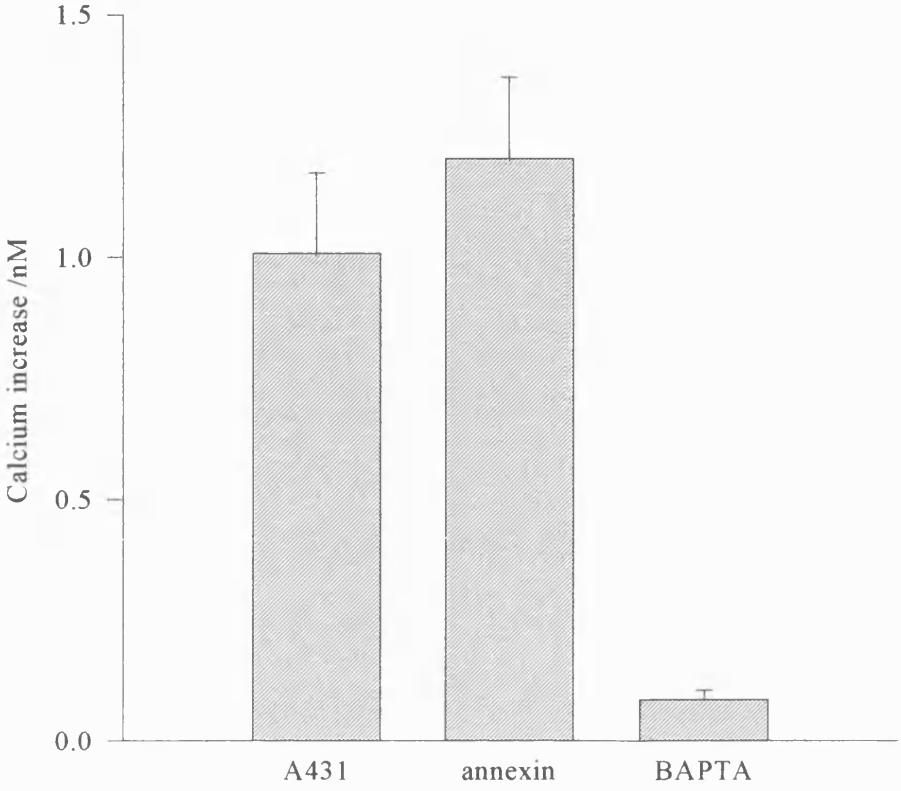


Figure 3.4.1: Comparison of wtA431 cells and annexin VI+ cells.

Both the wtA431 cells and the annexin VI+ cells show the same increase in free $[Ca^{2+}]i$ following an UV flash. Cells that were pre-loaded with BAPTA AM before injection show a negligible increase in $[Ca^{2+}]i$ following destruction of NP-EGTA.

DISCUSSION: CALCIUM BUFFERING CAPACITY OF N1E-115 AND A431 CELLS

4.1 N1E-115 CELLS

N1E-115 neuroblastoma cells were used as a neuronal model. The clone can be induced to differentiate with 2% DMSO (Kimhi *et al*, 1976). All cells were differentiated, and plated on Petriperms. Cells that had not yet extended neurites were selected for these experiments, because with these the volume of the cell could be estimated from simple cell dimensions. Although this remains an estimate, cell size was fairly standard, and so errors in cell volumes would be similar across the range of cells used.

The use of SR allowed the amount of injection solution introduced into each cell to be measured. SR was used as a marker since its fluorescence is unaffected by cell constituents. Thus, a comparison of the fluorescence measured from the cell, knowledge of the volume, and a standard calibration allowed the calculation of the amount of SR introduced into the cell. Since the injection solution had a constant ratio of SR:F:NP-EGTA, the total amount of calcium buffer that had been injected could be calculated.

The injection solution contained no added calcium, and so the calcium loading of the NP-EGTA was determined by the resting calcium concentration of the cells. [Ca:NP-EGTA] was calculated for each cell, and a plot of $\Delta[Ca^{2+}]_i$ (calcium released by photo-destruction of NP-EGTA) observed against [Ca:NP-EGTA] was produced (figure 3.3.1).

4.2 DESTRUCTION OF NP-EGTA

In order to calculate how much calcium has been buffered in these cells, the amount of NP-EGTA destroyed by a single UV flash had to be known, and had to remain constant. The simplest way of determining this would have been to measure the calcium released from a dilution of the injection solution where all concentrations of calcium buffers are known, when exposed to a single UV flash. All the increase in calcium seen would be due to photo-destruction of the NP-EGTA, and the amount binding to calcium buffers (the calcium indicator and remaining NP-EGTA) could be calculated, allowing a standard amount (%) of photo-destruction per flash to be assessed. However, this method of determining the intensity of the flash gun was unreliable and inconsistent, although it is not apparent why this should be so. It would appear that it relied heavily on the optical characteristics of the method used - solutions in small droplets gave different results to solutions contained in microcuvettes. Because of this inconsistency, the cells themselves were used as internal controls.

The problem with the use of the cells as an internal control is, of course, the fact that all the calcium buffering capabilities are not known. Although the concentrations of exogenous buffers can be accounted for, there is the endogenous buffering of the cell. In cells heavily injected with the injection solution, this endogenous buffering is masked by the exogenous buffers that have been introduced. The contribution to calcium buffering from the cell becomes negligible and therefore can be excluded. Since the total buffering capacity is therefore the exogenous buffers introduced, and the concentrations of these are known, the calcium released from the photo-destruction of NP-EGTA through a

single UV flash can be estimated. Therefore the data obtained were fit with an expression where there are two unknowns - the buffering capacity of the cell, and the percentage NP-EGTA destroyed in a single flash. This fit gave the values of 22% ± 8 for the percentage NP-EGTA destroyed with each flash and the endogenous buffering capacity of the cell, B, as 1707 calcium ions bound for every calcium ion free, ± 1455 (5% confidence level).

4.3 COMPARISON WITH OTHER RESULTS

Other work, principally on myocytes, but also including the neuronal work, place the buffering capacities of the cells far lower than the estimate produced from this work. Most other work places the buffering capacity of the cell as between 50 and 150 calcium ions bound for each one free, values that lie outside the 95% confidence limits of the calculated fit to the present data (252 to 3162).

It must be considered that these cells, as a cultured cell line, may not accurately mimic primary cells, and may have an abnormally increased calcium buffering capacity. The only way of determining whether this is the case would of course be to repeat the experimental method used here on other cells. There was no time to pursue that avenue.

Other reasons why the value calculated here is significantly higher than other values quoted may be as a result of the methods employed. In all other studies, the intracellular calcium in the cell is raised by opening calcium channels in the plasma membrane of the cell, allowing calcium influx. Methods of calcium removal by the cell (uptake into organelles or extrusion across the cell membrane) were eliminated where applicable, and CICR prevented. In general, the calcium

increases within the cell were large and located primarily at the periphery of the cell. The danger with this approach is that the calcium buffers at the periphery will be saturated by the calcium load, whereas the buffers further into the cell are far from their capacity. This may give the impression of a lower buffering capacity by the cell because the peripheral buffers have been saturated and so the increase in calcium concentration seen is higher.

In the method employed here, the calcium load was distributed throughout the cytoplasm and not located principally in any sub-area of the cell. Also, the amount of calcium released was very low - in the nM range rather than in the μM range, and so no cell calcium buffers were likely to be saturated by such a load.

It must be considered that the method of raising intracellular calcium in a neuronal cell would be through the opening of calcium channels in the plasma membrane in many circumstances *in vivo,* and so use of that method would not be physiologically inappropriate. As, physiologically, there exists this method of raising intracellular calcium, it would not be unreasonable to suggest that there would be a higher concentration of calcium-binding cell components located in this region. Therefore, it would be important, if, during the normal course of signalling, these buffers would be saturated, as this would affect the magnitude of the signal progressing into the centre of the cell. However, as a measurement of total calcium buffering within the cell, it is flawed.

The method employed here is unaffected by such sub-cellular localisations of buffers within the cell. It examined the buffering capacity of the cells without defining concentrations or Kds of any of the buffers, and looked purely at how many calcium ions would be bound for each one free. Also, no buffers, regardless of their sub-cellular localisation, concentration or Kd would be saturated by the

small increases in intracellular free calcium produced in these experiments. Thus, although the calcium load applied to the cell may be non-physiological, it is a more accurate way of assessing the total cytoplasmic calcium buffering than through driving calcium up at the periphery of the cell.

4.4 ANNEXIN VI

Is annexin VI acting as a calcium buffer?

The annexins are a family of proteins characterised by calcium-dependent phospholipid binding. As such, it might be expected that they would be calcium buffers - something that binds in a calcium-dependent manner obviously has calcium-binding sites. However, this may not be their principle role. Many functions have been suggested for the annexins, both individually and as a family. The role of annexin VI has been described variously as anything from a calcium channel (Pollard *et al*, 1992) through to having a role in endo- and exocytosis (Lin, Südhof and Anderson, 1992; review by Creutz, 1992; Smythe *et al*, 1994), and growth modulation (Theobald *et al*, 1994, 1995). Its role in calcium signalling following stimulation of cells with growth hormone is a negative one - the changes in intracellular calcium seen in cells transfected with and expressing annexin VI are attenuated in comparison with control cells (see section 5.2). The buffering capacities of annexin VI^+ cells and control cells were therefore compared, using the same technique as for the N1E-115 cells.

If the changes in $[Ca^{2+}]_i$ for a particular concentration of Ca:NP-EGTA are compared between the two cell types, there is no discernible difference seen in the observed calcium change (figure 3.4.1). Since the same amount of calcium is

released in each case from the NP-EGTA, an increased buffering capacity in the annexin VI^+ cells due to the presence of the protein would result in a decreased ΔCa^{2+} observed. Since this is not the case, it would appear that annexin VI is not acting purely as a calcium buffer, and the negative role in calcium signalling it plays must be through some other method.

To ascertain whether a simple comparison such as this was valid, or whether a full study of the full range of Ca:NP-EGTA concentrations was necessary (since only a comparison rather than a definite figure for buffering was desired), cells were loaded with the calcium buffer BAPTA. Thus not only were the endogenous buffers of the cell and the exogenous buffers from the injection solution present, but another calcium buffer was introduced. Again, comparing cells with the same concentration of Ca:NP-EGTA present (and therefore the same amount of calcium released) in the three groups showed that there was almost no increase in $[Ca^{2+}]_i$ in the cells loaded with BAPTA AM, whereas the control and annexin VI^+ cells both showed a calcium increase following the UV flash, and the increases were not significantly different from one another (figure 3.4.1). Hence, the presence of extra buffering in the cell would appear to be registered through a simple comparison of $\Delta[Ca^{2+}]i$, eliminating the need for a lengthy study to ascertain the buffering capacity numerically.

To summarise, the buffering capacity of a neuronal cell line, N1E-115, was ascertained as 1707 ± 1455 calcium ions bound to endogenous buffers of the cell for every free calcium ion in the cytoplasm. This corresponds to an intracellular

concentration of a single buffer whose Kd is that of the resting calcium concentration in these cells (45nM) of 306μM.

Annexin VI, a member of the calcium-dependent phospholipid binding family of proteins, which exerts a negative effect on cell growth in A431 cells, does not do so through being a significant calcium buffer, as determined by a comparison of $\Delta[Ca^{2+}]_i$ elicited in cells in response to calcium release from a caged calcium compound, NP-EGTA.

ANNEXIN VI

5.1 PRELIMINARY WORK

5.1.1 Western blots of cells

A431 cells were transfected with either pRC/CMV (control transfectants) or pRC/CMV.Anx6 (annexin VI^+ clones) in S.E. Moss' laboratory, as described in "Methods". All cell types were screened, in the same laboratory, to investigate the level of annexin VI expression, through Western blotting, as described in "Methods". As has been shown before (Theobald *et al*, 1994), neither the wild type parental A431 cells (wtA431) nor the clones transfected with the control plasmid expressed annexin VI, whereas the clones A6.1 and A6.2 were expressing annexin VI stably. The clone A6.1 expresses annexin VI at a medium level, similar or less than the expression in a human body cell, whereas the clone A6.2 expresses a high level of the protein.

5.1.2 Reduced growth rates

It has been demonstrated before that clones expressing annexin VI have reduced rates of growth, showing increased time to doubling in comparison with the parental A431 cells or control transfectants (Theobald *et al*, 1994). The difference is particularly striking when the cells are grown in low serum conditions. After a number of days, the cells expressing annexin VI reach confluency and become contact inhibited - a trait that is not shown by the wtA431 cells or the control transfectants. Analysis of the proportion of cells in the

different phases of the cell cycle indicated that a higher proportion of the annexin VI clones were growth arrested in G_1 than the control cells which showed a normal proportion of cells in each phase of the cell cycle.

5.2 CALCIUM SIGNALLING

5.2.1 Dose response curves for cell types to rhEGF

Wild type A431 cells, control transfectants and cells transfected with annexin VI and expressing different levels of the protein were perfused with varying concentrations of rhEGF, in normal saline. The peak calcium concentration attained by all cell types within five minutes after stimulation with rhEGF were recorded, and the results are shown in figure 5.2.1 as dose response curves for the cell types. The cells were perfused with varying concentrations from 10ng/ml to 100ng/ml rhEGF (maximum stimulation). The calcium concentrations corresponding to 350/380 nm fluorescence emission ratios were calculated as described in materials and methods and are shown on the right hand axis. It can be seen that maximum calcium concentrations are obtained in wtA431 cells when stimulated with rhEGF at a concentration of 50ng/ml, whereas cells expressing a medium level of annexin VI (A6.1) require stimulation with the highest concentration (100ng/ml) of rhEGF before attaining the equivalent peak calcium concentration. The clone heavily expressing annexin VI (A6.2) shows no significant increase in intracellular calcium concentration even when perfused with the highest concentration of agonist. The control transfectants C7 and C8 show a peak calcium concentration equivalent to the wtA431 when stimulated with the lower concentration of rhEGF (50ng/ml). Hence it would appear that

expression of annexin VI produces an impaired response to the growth factor - in other words the dose-response curve has been shifted to the right.

Since annexins have calcium binding properties, one possible explanation of these data is that the cells responded normally to the agonist, but that annexin VI mopped up the calcium so that it was not free in the cytoplasm. That this is not the case has been demonstrated by the results of experiments comparing the buffering capacities of wtA431 cells and annexin VI^{+} cells and has been discussed in chapter four. Further evidence against a simple buffer model comes from the patterns of response elicited in the different clones following stimulation with the growth factors EGF and BK, described below.

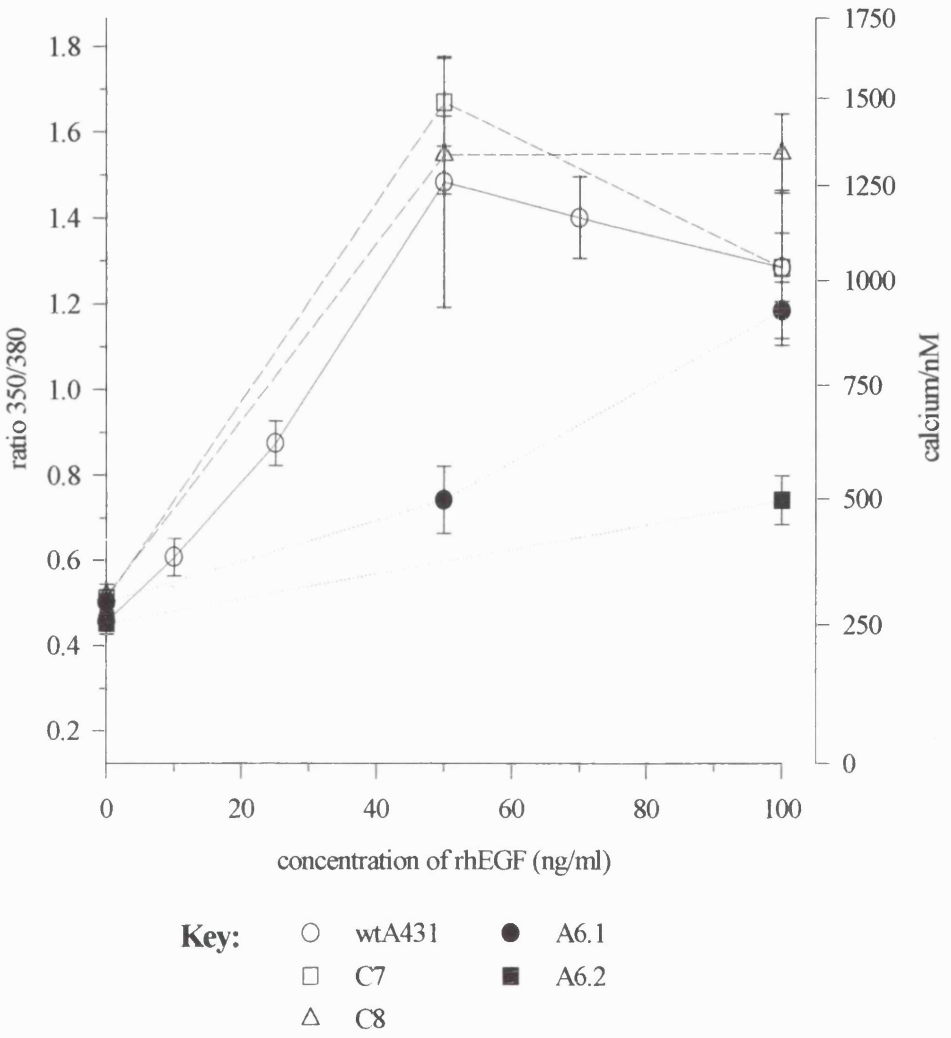


Figure 5.2.1: Dose response curve for the cell types.

wtA431, control transfectants (C7 and C8) and annexin VI+ cells (A6.1 and A6.2) were stimulated with concentrations of rhEGF from 10-100ng/ml as indicated, and the maximum ratio ($[Ca^{2+}]i$) over the following five minutes was recorded. Each point represents the mean ratio ($[Ca^{2+}]i$) and error bars indicate SEM. N is between 20 and 89 (median 65) from a minimum of 3 platings, except A6.1; 50ng/ml which is from 2 platings.

5.2.2 Sources of calcium in the response to rhEGF

It is known that there are two sources of calcium in the response to rhEGF by wtA431 cells. The initial peak of raised intracellular calcium is the release from intracellular stores through activation of $InsP_3$ receptors on intracellular calcium stores (Hughes *et al*, 1991; Wheeler, Goodrum and Sachs, 1990; Pandiella *et al*, 1987). However, it has been shown that the following plateau of increased calcium concentration within the cell is dependent on extracellular calcium levels and is due to the influx of calcium across the plasma membrane, rather than release from intracellular sources.

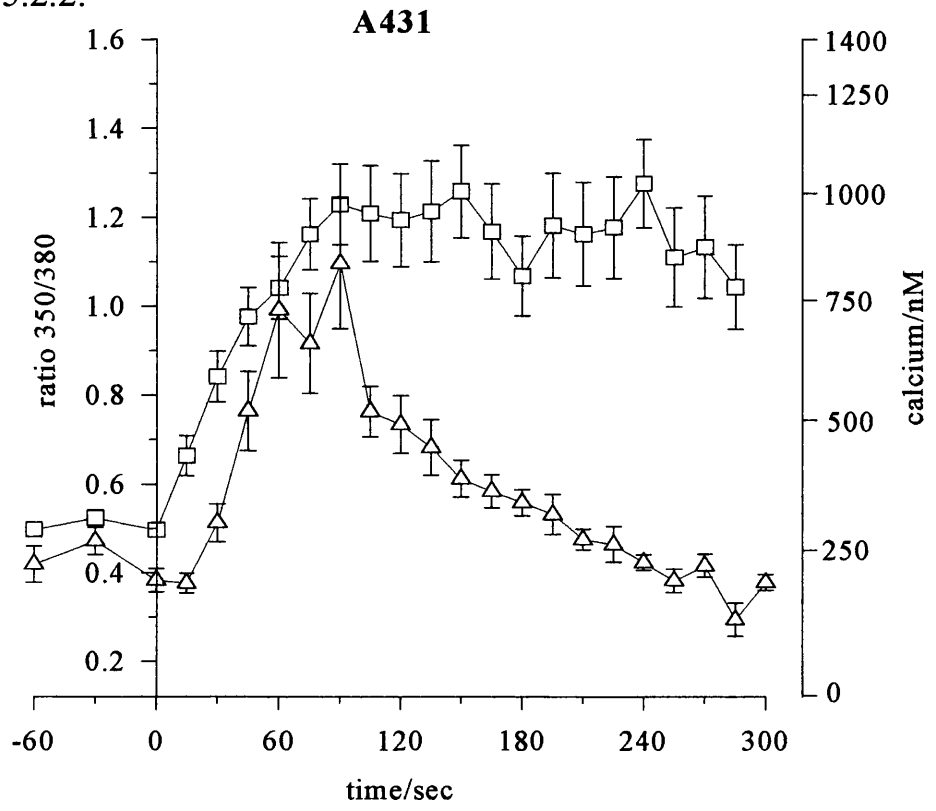
The plateau results from influx of calcium across the plasma membrane, so the responses of wtA431 cells, control transfectants and annexin VI^+ cells were examined when stimulated with rhEGF (100ng/ml) in the presence and absence of extracellular calcium. The results are shown in figure 5.2.2 (note that the peaks in these traces represent the time point at which the mean ratio from all cells was maximal, and is therefore likely to be smaller than the value plotted at the right of figure 5.2.1, which represents the peak value in each cell, whenever it occurred, averaged). The wtA431 cells and control transfectants show a marked plateau of raised intracellular calcium concentration from entry of external calcium - the trace for the response in calcium-free saline falls rapidly back to resting levels after the initial peak, whereas in normal, calcium-containing saline, the initial peak is followed by a calcium concentration well above baseline for many minutes (figure 5.2.2 a-c).

However, the medium expression annexin VI^+ cells (clone A6.1) show no such maintained calcium increase above baseline values beyond the peak when

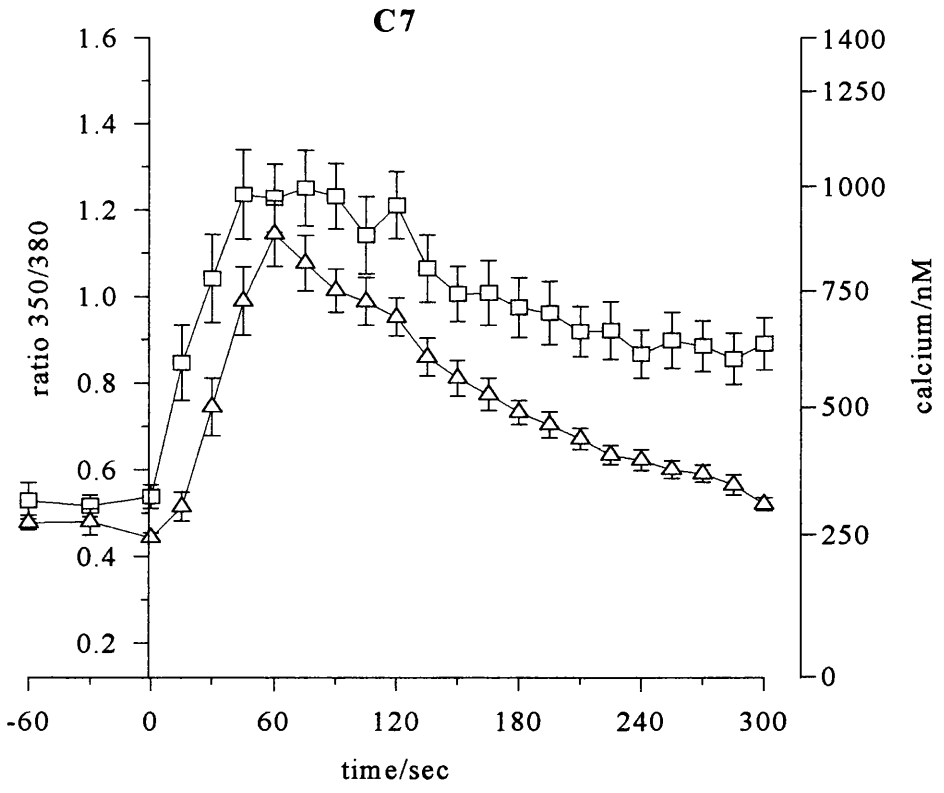
stimulated in calcium-containing saline. Indeed, the two traces (in normal HBS and in Ca-free HBS) for the clone A6.1 very nearly track one another, indicating little, if any calcium influx in normal saline (figure 5.2.2d). As noted above, the heavy expression annexin VI^+ cells (clone A6.2) show no calcium response even to 100ng/ml rhEGF (figure 5.2.2e)

Figure 5.2.2:

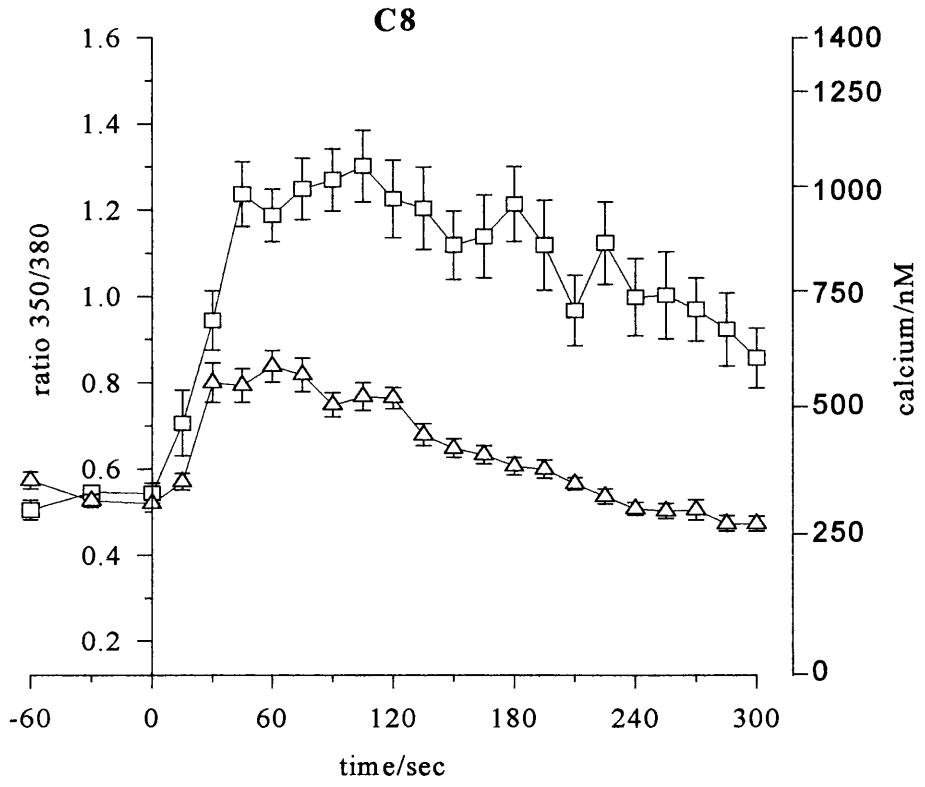
(a)



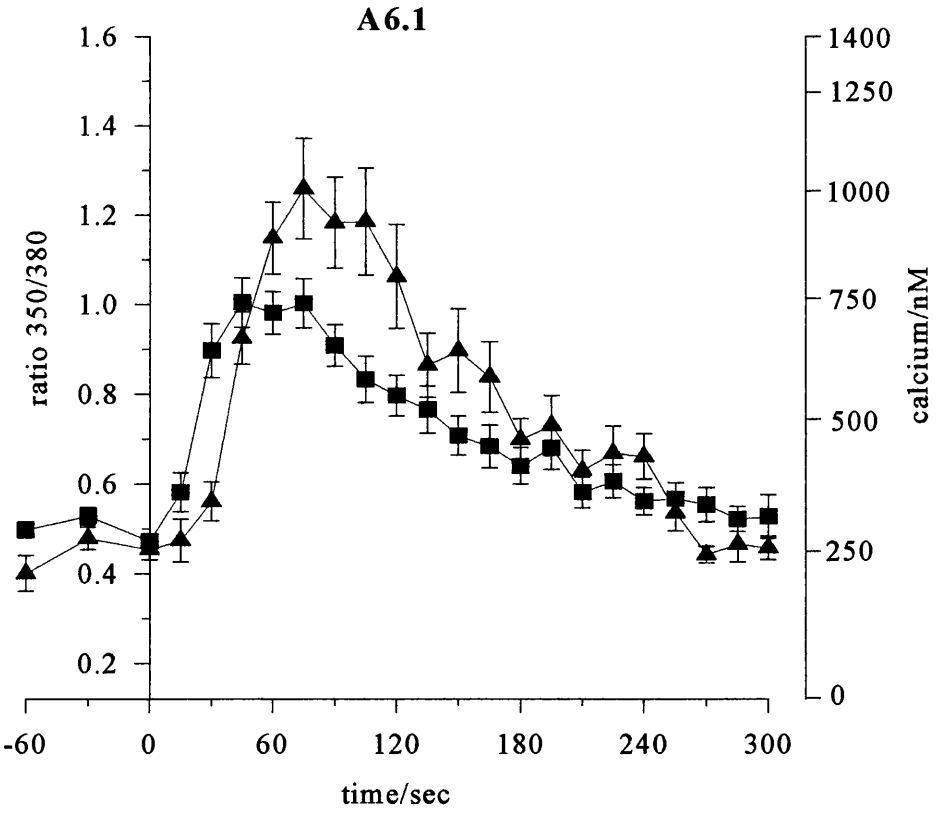
(b)



(c)


C8
ratio 350/380
calcium/nM
time/sec
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
1400
1250
1000
750
500
250
0
-60
0
60
120
180
240
300

(d)


A6.1
ratio 350/380
calcium/nM
time/sec
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
1400
1250
1000
750
500
250
0
-60
0
60
120
180
240
300

(e)

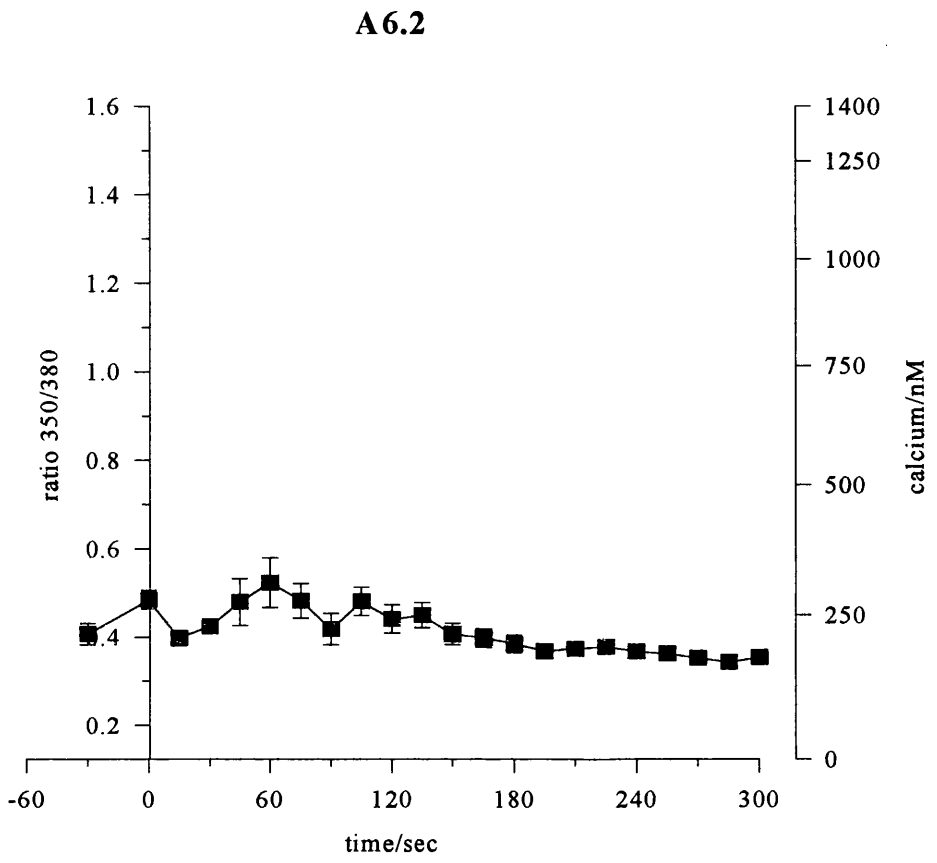


Figure 5.2.2: Time course of response to 100ng/ml rhEGF stimulation.

In each case, squares (filled or open) indicate experiments done in the presence of external calcium; triangles (filled or open) indicate experiments done in the absence of external calcium. Open symbols indicate clones which do not express annexin VI; filled symbols indicate clones expressing annexin VI. For each graph, N is between 23 and 89 cells (median = 51, minimum of three platings) and points indicate mean +/- SEM.

(a) wt A431 cells; (b) and (c) control transfectants; (d) A6.1 cells (medium level of annexin VI expression); (e) A6.2 cells (high level of annexin VI expression).

5.2.3 Neomycin

It can be seen from figure 5.2.2 that the control transfectants C7 and C8 have a slightly impaired plateau response to rhEGF compared to the response of the wtA431 cells. The response is, however, still significantly different from that of the annexin VI^+ cells. The control transfectants and the annexin VI^+ cells were cultured in media containing 200μg/ml neomycin, whereas the wtA431 cells were cultured in the absence of neomycin. To determine whether the presence of neomycin in the media was producing a slightly impaired response to rhEGF, a sample of control transfectants C7 were maintained in culture in the absence of neomycin for a week, by which time all the original cells subbed should have divided. These cells were then stimulated with 100ng/ml rhEGF in the presence and absence of extracellular calcium as described. The results are shown in figure 5.2.3. As before, the x-axis indicates time, the left hand y-axis indicates the ratio of 350/380 nm fluorescence emission recorded and the right hand y-axis the corresponding calcium concentration. The response of C7 cells after a period of week in the absence of neomycin is comparable to the responses recorded from wtA431 cells (figure 5.2.2a). Neomycin acts on prokaryotic cells by inhibiting protein translation by binding to the small subunit of the ribosomes. However, it also is a major inhibitor of inositol phospholipid turnover by inhibiting PLC and PLD. It is likely that some impairment of function of the cells is occurring.

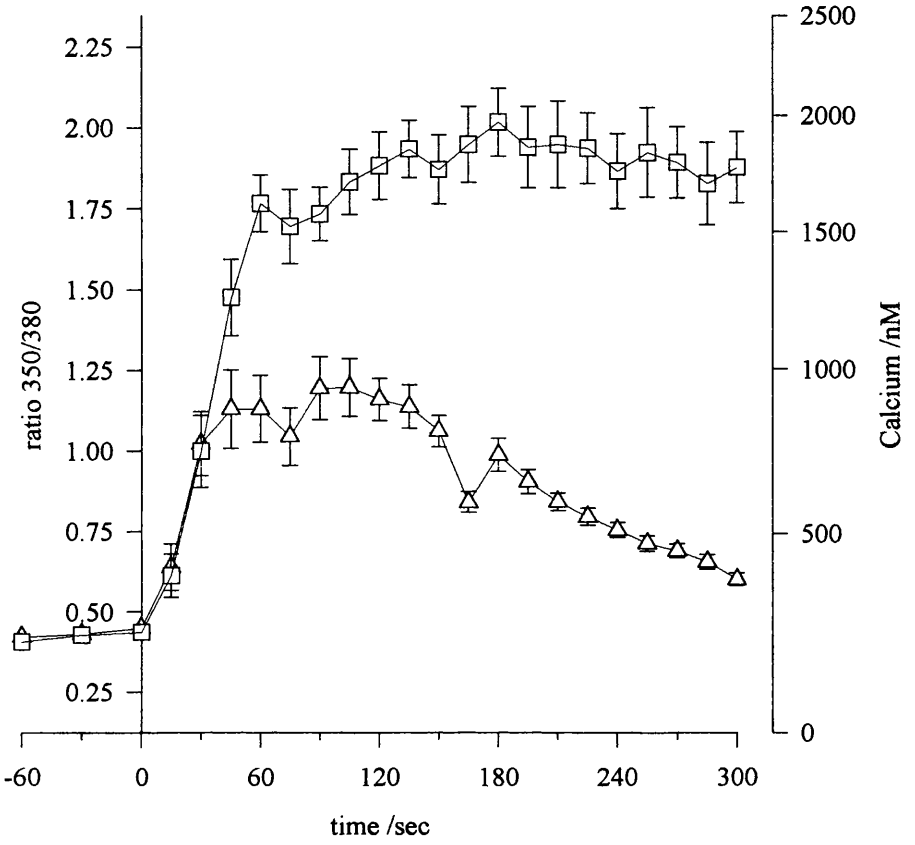


Figure 5.2.3: Response of C7 cells to rhEGF after seven days out of neomycin.

C7 cells (control transfectants) were stimulated with 100ng/ml rhEGF and the response over the following five minutes was recorded. The cells had been cultured in the absence of neomycin for seven days prior to the experiment. Squares represent experiments done in the presence of calcium in the saline, triangles indicate the absence of calcium in the saline. Each point represents the mean ratio for the time bin (15s) and error bars indicate +/- SEM. The right hand side y-axis represents the $[Ca^{2+}]i$ corresponding to the ratio. N = 35 for Ca+, and 42 for Ca-free. One plating.

5.2.4 Manganese quench

If it is the case that there is little or no influx of calcium across the plasma membrane when annexin VI is expressed by the cells, then a difference in the quench rate of Fura-2 by Mn^{2+} should be evident. Mn^{2+} ions bind to the dye Fura-2 and form a non-fluorescent complex. The greater the amount of Mn^{2+} present in the presence of Fura-2, the greater the loss of the fluorescent species. This quenching of the dye can be monitored by following the fluorescence intensity during excitation with the calcium-insensitive wavelength of the dye (360 nm). Under these conditions, the changes in fluorescence intensities caused by calcium will be eliminated and only the changes caused by the presence of Mn^{2+} will be monitored.

Figure 5.2.4(a) shows the baseline quenching of Fura-2. A6.1 and C7 cells were maintained in normal saline and the fluorescence intensity of the dye at its calcium-insensitive wavelength (360 nm) was monitored for three minutes. After this, (t = 180 s) the cells were perfused with saline containing 0.5mM $MnCl_2$, and monitored for a further five minutes. The data were normalised to the fluorescence intensity at the onset of the experiment. The baseline quench rate is not different in the two cell lines (Table 5.2.4).

Mn^{2+} ions enter the cytoplasm from Mn^{2+} supplemented saline through open calcium channels, regardless of the mechanism of opening of the channels - whether voltage- or agonist-gated. Hence, if the cell-types are stimulated with rhEGF in the presence of Mn^{2+}, and there is a difference in the ability of Mn^{2+} to enter the cytoplasm, either through the Ca^{2+}-channels not opening or the channels being blocked, the rate at which Fura-2 is quenched will differ between the two

cell types. Figure 5.2.4(b) shows the effect of stimulating the cell types A6.1 and C7 with 100ng/ml rhEGF on manganese influx. The fluorescence intensity of the dye at its calcium-insensitive (iso-fluorescent) wavelength (360 nm) was monitored for three minutes. After this, (t = 180 s on the graph) the cells were stimulated with 100ng/ml rhEGF, in the presence of 0.5mM $MnCl_2$, and monitored for a further five minutes. The data were normalised to the fluorescence intensity at the onset of the experiment. In the presence of EGF the quench rate is greater in the C7 cells as compared to the A6.1s (table 5.2.4). The cells expressing annexin VI, which showed impaired calcium influx across the membrane when stimulated with rhEGF, showed a slower rate of quench of Fura-2 fluorescence. Using analysis of variance (ANOVA) the difference between the group means is highly significant ($p < 0.0001$). More importantly, applying post-tests to look for differences between individual pairs of groups, applying Bonferronis correction one finds that C vs. D is significant - i.e. EGF does give extra influx in control cells. A vs. B is not significant - i.e. EGF does not affect influx in annexin expressors. Taken together, the manganese quench data and the data comparing responses in the presence and absence of external calcium, provide strong evidence that calcium influx following agonist application is seriously impaired in annexin VI^+ cells.

Table 5.2.4: Comparison of quench rates.

CELLS	% QUENCH AFTER FIVE MINUTES	
	- EGF	+ EGF
Control (C7)	34.38 ± 3.26 (N = 49) (**C**)	41.63 ± 3.37 (N = 50) (**D**)
Annexin VI^+ (A6.1)	31.34 ± 1.92 (N = 59) (**A**)	26.20 ± 2.68 (N = 42) (**B**)

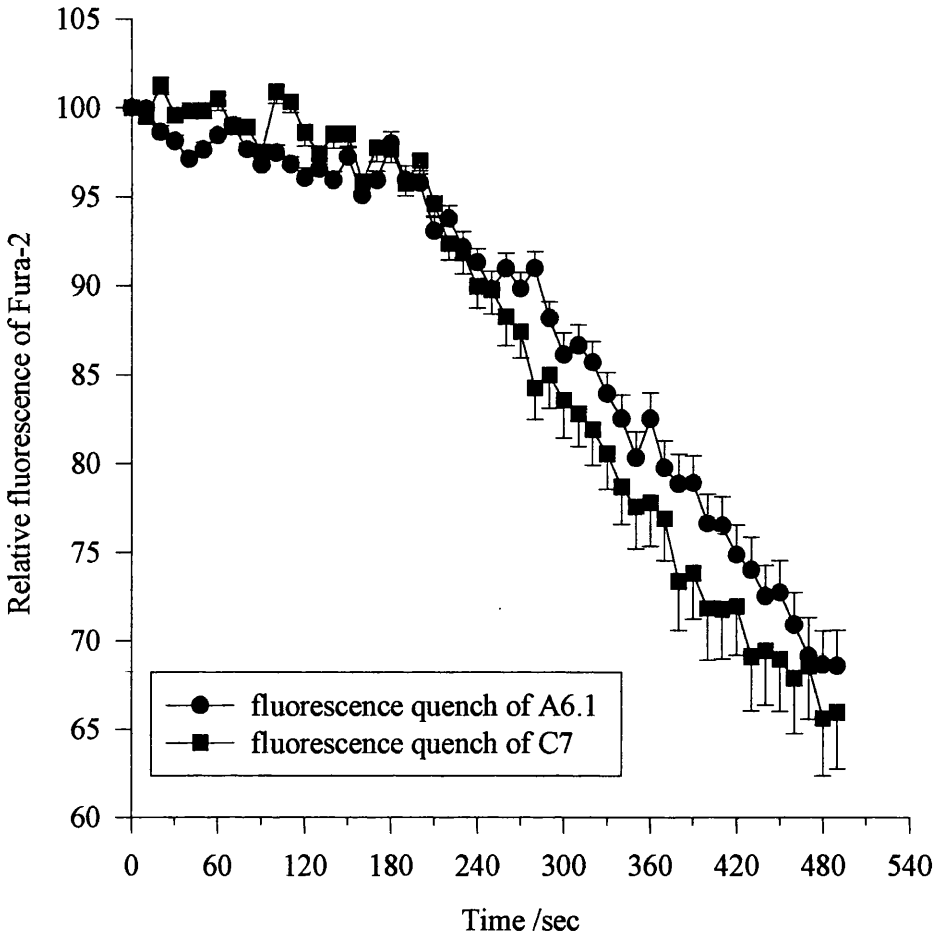


Figure 5.2.4 (a) Baseline quench of Fura-2.

Cells were maintained in normal saline for three minutes, then perfused with saline supplemented with 0.5mM $MnCl_2$. The fluorescence emission of Fura-2 excited with 360nm light was monitored to assess the extent of quenching. There is no difference in baseline quench between the annexin VI+ cells and the control transfectants. Points indicate mean +/- SEM of data that have been normalised to initial fluorescence intensity. N for A6.1 cells is 59 from 3 platings. N for C7 cells is 49 from 3 platings.

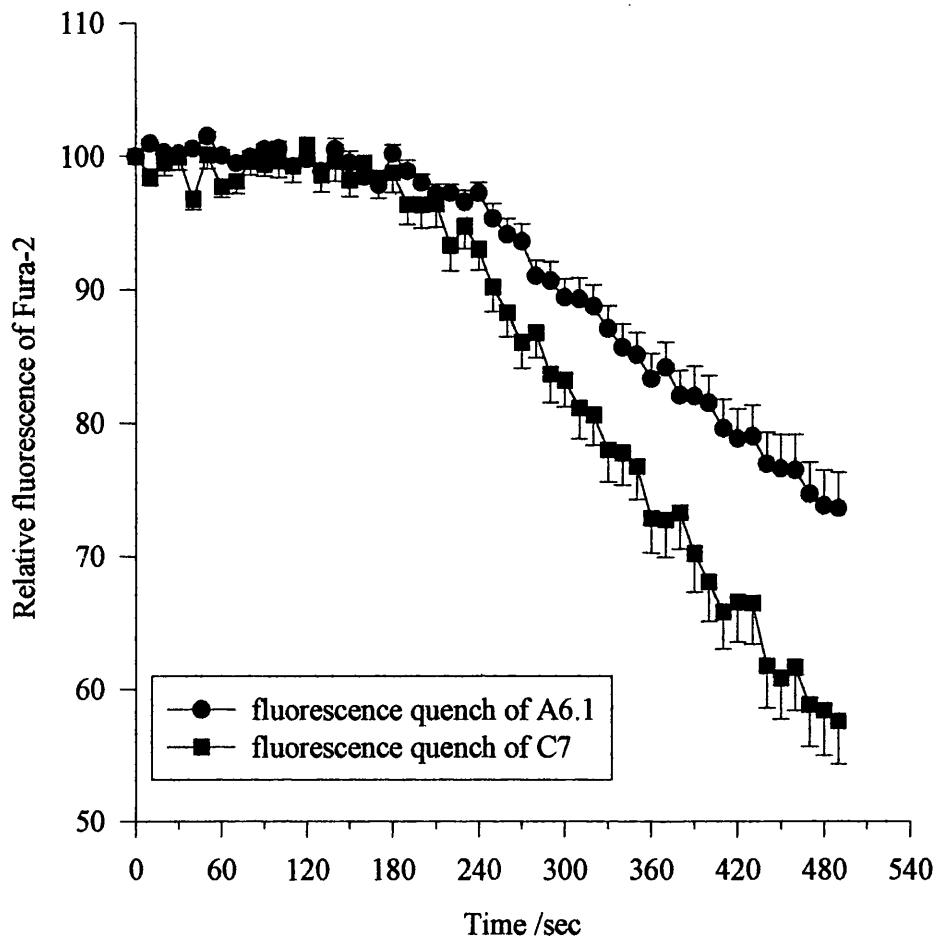


Figure 5.2.4 (b): Fura-2 quench during agonist application.

Cells were maintained in normal saline for three minutes, then perfused with saline supplemented with 0.5mM $MnCl_2$ and 100ng/ml rhEGF. The fluorescence emission of Fura-2 excited with 360nm light was monitored to assess the extent of quenching. The annexin VI+ cells show a reduced rate of quench in the presence of agonist when compared with control transfectants. Points indicate mean +/- SEM of data which have been normalised to initial fluorescence intensity. N for A6.1 cells is 42 from 3 platings. N for C7 cells is 50 from 3 platings.

5.2.5 Calcium store size in annexin VI$^+$ cells

Does annexin VI expression affect only EGF-R signalling, or is the signalling pathway activated by the seven transmembrane receptors/heterotrimeric G-proteins affected as well? The initial peak of raised intracellular calcium concentration is the largest calcium change seen after stimulation with agonist and is hence the value plotted against dose in figure 5.1.2. The source of calcium for this peak is the intracellular stores releasing calcium in response to a rise in intracellular $InsP_3$ levels. It is possible that the magnitude of these stores is reduced in annexin VI$^+$ cells, accounting for the shift in the dose response curve to the right. Not all the calcium released by the stores will be taken back up into the stores, rather, a small fraction would be expected to be pumped out of the cell into the extracellular fluid, replaced by some of the calcium that enters across the plasmalemma during the plateau phase. It has been demonstrated that the calcium influx following stimulation with agonist in cells expressing annexin VI is impaired, so it does not seem unreasonable to suggest that the internal stores of calcium may become depleted with time. With this in mind, it was decided to try and assess the size of the intracellular stores by releasing them entirely into the cytoplasm and measuring the calcium changes observed. However, this was not possible through the use of thapsigargin (see "Discussion", section 7.2.2). A comparison of the responses of the annexin VI$^+$ clones to bradykinin (BK) to the response of control transfectants to the same dose was made.

Bradykinin activates PLCβ and causes a release of calcium ions from intracellular stores with no measurable influx across the plasmalemma (Wheeler, Goodrum and Sachs, 1990; Pandiella *et al*, 1987). The Ca^{2+} increase on maximal

BK stimulation will therefore give a measure of intracellular store filling assuming the signalling pathway to $InsP_3$ production is intact. These results are shown in figure 5.2.5 a, b. Since BK also releases calcium from internal stores through activation of the $InsP_3$-R, but the PLC isozyme differs in the EGF signalling pathway, this will indicate whether the differences displayed by the annexin VI expressing cells are through an alteration at the EGF-R level or at the store level. If the responses are comparable between the cell types, it would imply that the stores are normal in the annexin VI^+ cells and that the difference lies at receptor level. If the responses are impaired, a number of possibilities could be responsible, including reduced store magnitude, direct interaction and/or interference by annexin VI in both the RTK-mediated and heptahelical/G-protein-mediated PIP_2 hydrolysis pathways or that annexin VI could be affecting the pathways indirectly through binding to the phospholipids of the pathways in a calcium-sensitive fashion.

Figure 5.2.5(a) gives a comparison of the peak response to 1μM BK in the four minutes following agonist application, for cell types A6.1 (medium annexin VI expression), A6.2 (heavy annexin VI expression) and C1 (control transfectants). Both the A6.1 and A6.2 clones show an impaired response to BK in comparison with the control transfectants, although this difference is only significant for A6.1 clones ($p < 5\%$, ANOVA). For each cell type, the open portion of the bar indicates the mean ratio before application of BK. The overall magnitude of the bar indicates the mean peak ratio measured over the four minutes following agonist perfusion. Error bars indicate SEM and N is given in the figure legend.

Figure 5.2.5 (b) shows the time course of the response following agonist stimulation. The data have been binned into 15 second time bins (as indicated) and the mean ratio for each bin calculated. Points represent mean ratio; error bars indicate SEM. All clones show the same pattern of response, but the peak $[Ca^{2+}]_i$ attained by both of the annexin VI^+ cells is attenuated. These results are discussed in Chapter 7, following presentation of the experiments addressing the question of cross-talk between the EGF and BK activated pathways.

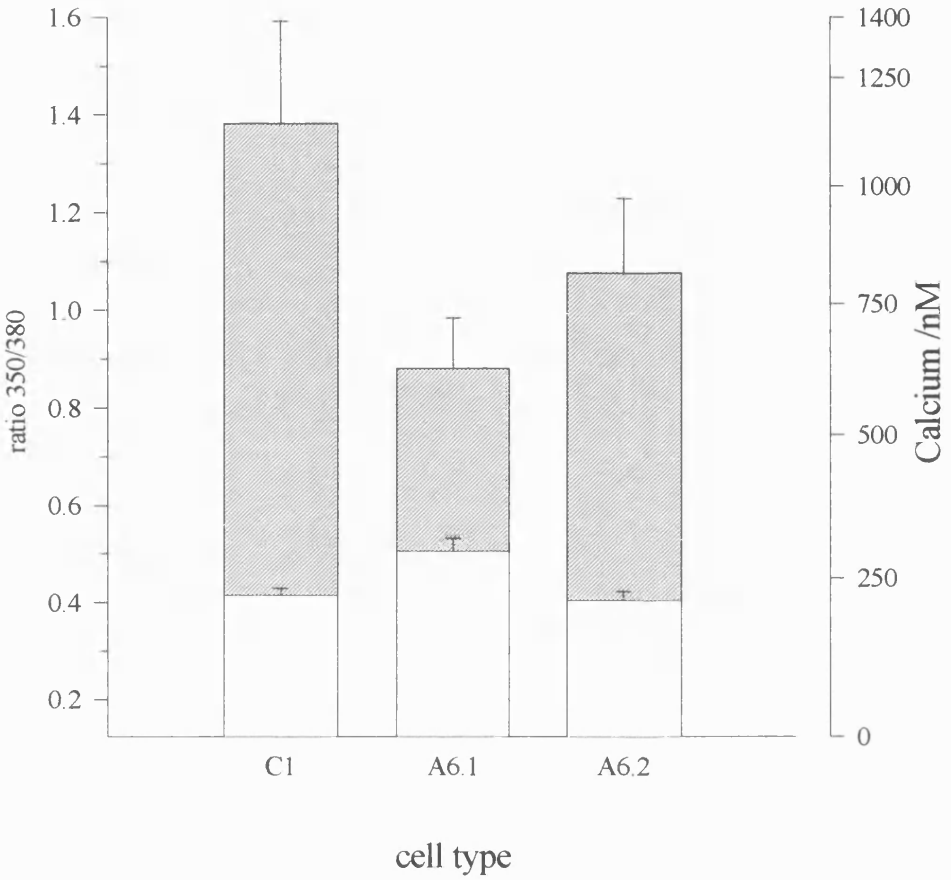


Figure 5.2.5: Comparison of responses to 1μM bradykinin (BK).

(a) Cells were stimulated with 1μM BK and the peak ratio following stimulation measured. The left-hand y-axis indicates the mean peak ratio attained. The shaded portion of the bar represents mean resting ratio; the open portion of the bar represents mean peak ratio for each cell type. The right-hand y-axis indicates corresponding $[Ca^{2+}]i$. Both the clones expressing annexin VI show an impaired response to 1μM BK. N for C1 cells is 21, for A6.1 cells is 36 and A6.2 is 24 from a minimum of 3 platings in each case.

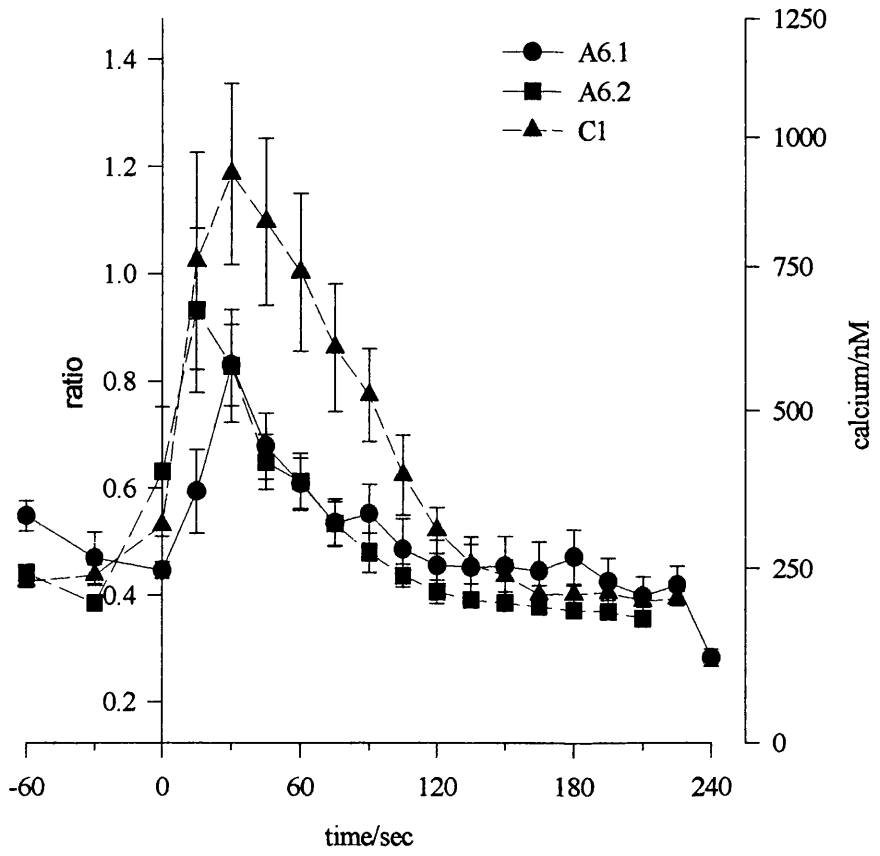


Fig 5.2.5 (b): Time course of the response to 1μM BK.

All clones show the same pattern of response, but the peak $[Ca^{2+}]i$ attained by the annexin VI+ cells is attenuated. Points represent mean ratio for the time bin (15s) and error bars indicate SEM. Right hand side axis indicates $[Ca^{2+}]i$ corresponding to the ratio. N for A6.1 = 36, for A6.2 = 24 and for C1 = 21, from a minimum of 3 platings in each case.

CROSS-TALK

6.1 PRELIMINARY WORK

6.1.1 Response of wtA431 cells to rhEGF

wtA431 cells were cultured and loaded with Fura-2 AM as described. The cells were then rinsed and maintained in saline and resting ratios obtained. After approximately 60 seconds, the cells were perfused with saline containing 100ng/ml rhEGF and maintained in this saline for a further five minutes, during which time pairs of images for the calculation of ratios (and thus calcium concentrations) were taken approximately every 15 seconds.

Figure 6.1.1 shows the time course for the calcium response to rhEGF. The x-axis denotes time in seconds, the left hand y-axis the ratios obtained from the cells and the right hand y-axis the calcium concentration corresponding to the ratios, calculated as described in section 2.2.1q. Application of the growth factor is presented as t = 0, so pre-treatment times are denoted by negative times. The data has been collected from 154 cells (>3 platings) and binned into 15-second time bins. The average ratio for all the cells within the time bin has been calculated and the error bars on the graph indicate SEM.

It can be seen that the calcium concentration within the cell rises rapidly, reaching a peak after about 60 seconds. The calcium concentration then levels off, or plateaus for the remainder of the five minutes' observation. Thus, the response to rhEGF has two phases, a rising phase followed by a maintained plateau. The early response is unaffected by extracellular calcium concentrations (Hughes *et al*, 1991; Wheeler, Goodrum and Sachs, 1990; Pandiella *et al*, 1987 and see also

figure 5.2.2a) and is due to release of calcium from internal stores. The plateau is affected by extracellular calcium concentration (Hughes *et al*, 1991; Wheeler, Goodrum and Sachs, 1990; Pandiella *et al*, 1987 and see figure 5.2.2a) and is due to influx of calcium across the plasma membrane.

When EGF binds to the EGF-R, PLCγ is activated through tyrosine phosphorylation. This promotes the hydrolysis of PIP_2 to DAG and $InsP_3$ and the $InsP_3$ causes release of calcium from internal stores. This produces the initial increase in free calcium concentration in the cell. Through a still somewhat largely undefined pathway, EGF-R activation with high levels of EGF leads to influx of calcium across the cell membrane.

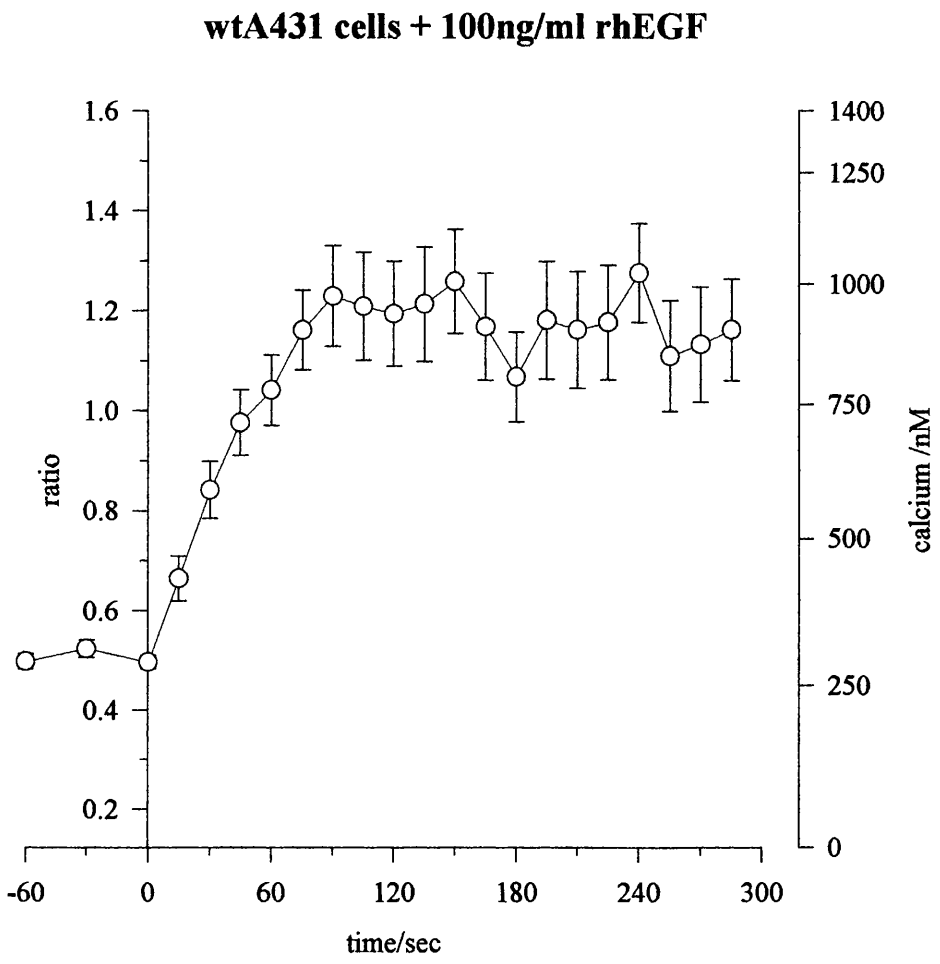


Figure 6.1.1: Response of wtA431 cells to 100ng/ml epidermal growth factor (rhEGF).

Cells were stimulated with 100ng/ml rhEGF at t = 0. The response to the growth hormone shows two phases; a rapid rise in calcium, followed by a slight fall to a new, maintained plateau level of calcium. N = 154 from >3 platings.

6.1.2 Response of wtA431 cells to BK

wtA431 cells were cultured and loaded with Fura-2 AM as described. The cells were then rinsed and maintained in saline and resting ratios obtained. After approximately 60 seconds, the cells were perfused with saline containing 1μM BK and maintained in this saline for a further five minutes, during which time pairs of images for the calculation of ratios (and thus calcium concentrations) were taken approximately every 15 seconds.

Figure 6.1.2 shows the time course for the calcium response to BK. The x-axis denotes time in seconds, the left hand y-axis the ratios obtained from the cells and the right hand y-axis the calcium concentration corresponding to the ratios, calculated as described in section 2.2.1q. Application of the agonist is presented as t = 0, so pre-treatment times are denoted by negative times. The data has been collected from 348 cells (>3 platings) and binned into 15-second time bins. The average ratio for all the cells within the time bin has been calculated and the error bars on the graph indicate SEM. This dataset differs from that of figure 5.2.5 in that the cells being stimulated with agonist are wtA431 cells, that have not been transfected with annexin VI or control plasmid, therefore they have not been in the presence of neomycin.

It can be seen that $[Ca^{2+}]_i$ rises sharply after application of 1μM BK and then rapidly returns to baseline values. There is no prolonged raised calcium level as part of the response to BK. The sharp peak is not dependent on extracellular calcium concentration and again is the result of the release of calcium from internal stores as a response to raised levels of $InsP_3$ in the cytoplasm, following PIP_2 hydrolysis. The enzyme responsible for the hydrolysis of PIP_2 in this case is

PLCβ, and the interaction between the BK-R and PLCβ is through a G-protein, rather than through direct phosphorylation (Wheeler, Goodrum and Sachs, 1990; Pandiella *et al*, 1987).

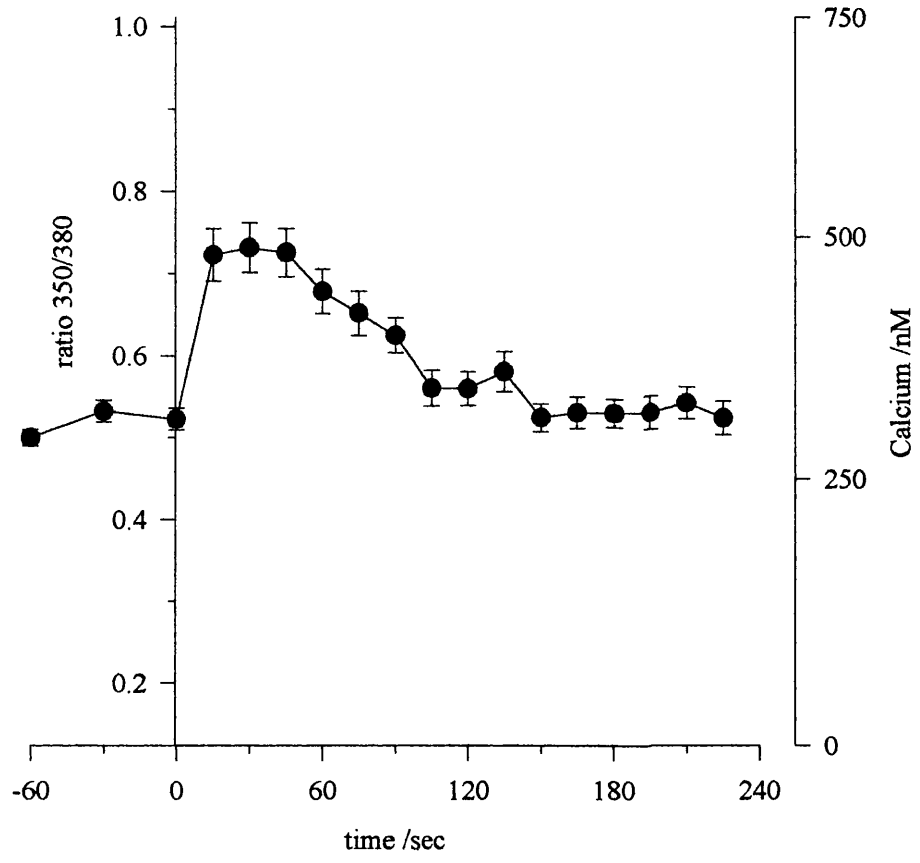


Figure 6.1.2: Response of wtA431 cells to 1μM bradykinin (BK).

Cells were stimulated with 1μM BK at t = 0. The response is monophasic; there is a sharp rise in calcium that falls to resting values rapidly. No maintained plateau of raised calcium is observed. N = 348, from >3 platings.

6.2 CROSS-TALK BETWEEN THE RECEPTORS

Previous work on the interaction between receptors examining phosphorylation of the receptors (Hosoi, Kurihara and Ueha, 1993) or components of a signalling pathway (Liebmann *et al*, 1996) have demonstrated potential mechanisms for signal modulation, both at the receptor level (through alterations in the affinity of a receptor for its ligand) and downstream. However, the physiological effects of such modulation in the whole cell have not been examined. In this thesis, the effect of potential interaction on calcium signalling was examined, having established the control response (i.e. with no prior stimulation with another agonist).

6.2.1 Response of wtA431 cells to rhEGF following prior BK stimulation

Both BK and EGF trigger production of $InsP_3$ that in turn causes an initial increase in intracellular calcium. There is therefore the possibility that prior exposure to one agonist might affect the subsequent response to the other by, for instance, partially emptying the intracellular calcium stores. However, biochemical and pharmacological evidence suggests the possibility of longer lasting and more complex interactions. It is known that phosphorylation states of the receptors can affect their binding affinities (Hosoi, Kurihara and Ueha, 1993) and that the other hydrolysis product of PIP_2, DAG can affect PKC, and ultimately affect the phosphorylation of many proteins, including the membrane receptors to these agonists. Prior stimulation with BK may shift the dose response curve of wtA431 cells for EGF to the right, through this phosphorylation mechanism, although BK has a biphasic effect on the EGF-R through the additional action of a

phosphoprotein phosphatase. The dephosphorylation leads to an increased affinity of the receptor for EGF.

To examine this, wtA431 cells were cultured and loaded with Fura-2 AM as described. The cells were rinsed and maintained in saline and resting ratios taken. After approximately 60 seconds in some of the experiments, the cells were perfused with saline containing 1μM BK for 60 seconds. In other experiments, there was no prior stimulation with BK. After the 60 seconds in 1μM BK, the cells were rinsed with fresh saline for 180 seconds, then perfused with saline containing differing concentrations of rhEGF from 10 - 100ng/ml. Figure 6.2.1 shows the dose response curves for the response of wtA431 cells with or without prior stimulation with BK. The dotted line indicates the dose response curve with no prior BK; the solid line indicates the dose response curve following prior stimulation with BK. For each cell, the peak 350/380 ratio in the five minutes following rhEGF application was determined. These values were then averaged to give the mean peak for a particular stimulation protocol. Each point represents mean ± SEM. N for each point is given in the figure legend. All the data presented are paired - that is, control and pre-treatment regimes were performed on cells from the same plating.

Pre-treatment with 1μM BK has shifted the dose response curve to the left - the response of A431 cells to rhEGF following prior exposure to BK is greater at low concentrations of EGF (25, 50ng/ml) than the response seen in naïve cells. Unpaired t-tests between the control and pre-treated data show that the response to 25 and 50ng/ml EGF is significantly greater in pre-treated cells ($p = 0.0025$ and

0.047 respectively; Instat). The response to the maximum dose of EGF however, is significantly lower in the pre-treated cells ($p = 0.047$; unpaired t-test, Instat).

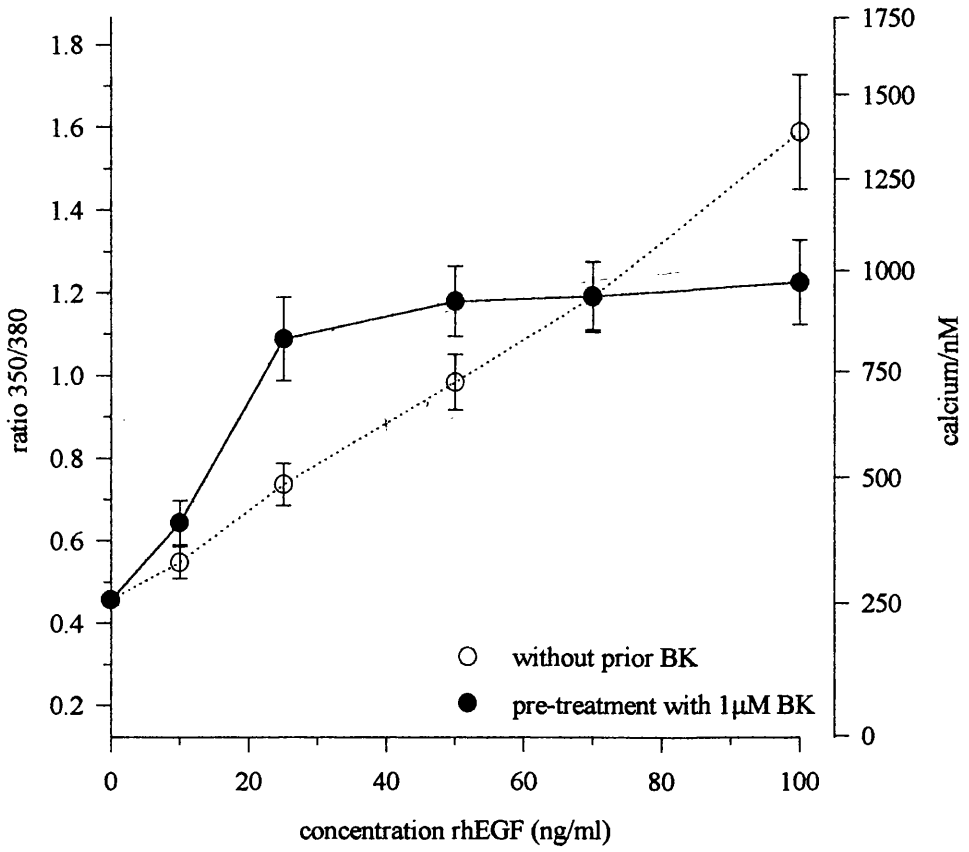


Figure 6.2.1: Response to rhEGF following prior stimulation with BK.

Cells were stimulated with 1mM BK for 60s, followed by 180s washout with HBS, then five minutes of perfusion 10-100ng/ml rhEGF as indicated. Open circles indicate the mean peak ratio ($[Ca^{2+}]i$) to the rhEGF dose with no prior BK, filled circles indicate the mean peak ratio ($[Ca^{2+}]i$) to the rhEGF dose with prior BK. N are as follows: no prior BK, 10-100ng/ml respectively - 51, 64, 58, 67, 78; with prior BK, 10-100ng/ml respectively - 50, 66, 74, 71, 60 from a minimum of 3 platings in each instance.

6.2.2 The pattern of response to rhEGF

The response to rhEGF has two phases - an initial rise in calcium corresponding to release from intracellular stores and a secondary phase due to influx across the plasma membrane (Wheeler, Goodrum and Sachs, 1990; data presented here).

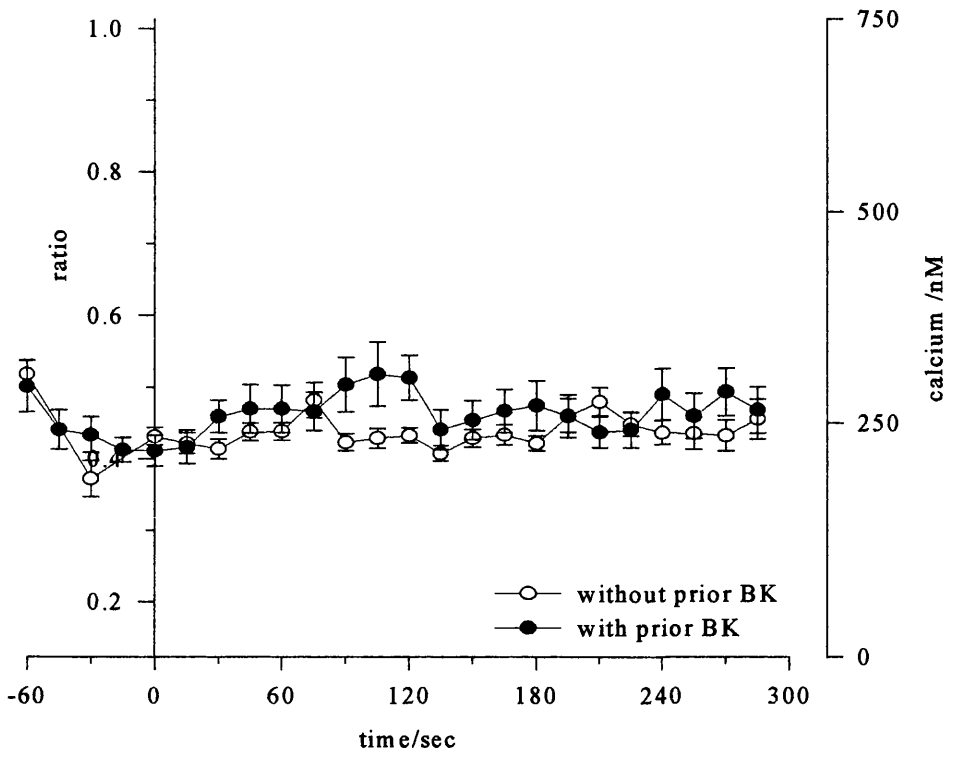
Both the patterns of response and the peak calcium response have been examined in this study. Figure 6.2.2 (a-e) shows the average response to a range of rhEGF concentrations with or without prior BK. Each symbol represents the mean ratio for all cells at that time point. Error bars indicate SEM. The right hand y-axis gives the equivalent $[Ca^{2+}]_i$ for the ratio. In each graph, open circles indicate no prior BK, filled circles indicate prior BK treatment. The data presented here are the same paired data as for figure 6.2.1.

The slight mismatch between the peak calcium concentration indicated in figure 6.2.1 and the peak calcium concentration indicated in figure 6.2.2 is a result of variability in the cells in the delay before responding to EGF. In figure 6.2.2 the data from individual cells are averaged in time, whereas in figure 6.2.1 the peak ratio over the entire five minutes after EGF application is determined and this value then averaged over all cells. There is no clear time point at which the majority of the cells are at the maximum calcium concentration for either treatment condition and so both control and pre-treated data appear flattened with respect to the peaks in figure 6.2.1. This flattening of the time-averaged data occurs across the range of pre-treatment doses. It was observed by Cheyette and Gross (1991) that the time to peak reduced with increasing agonist concentration

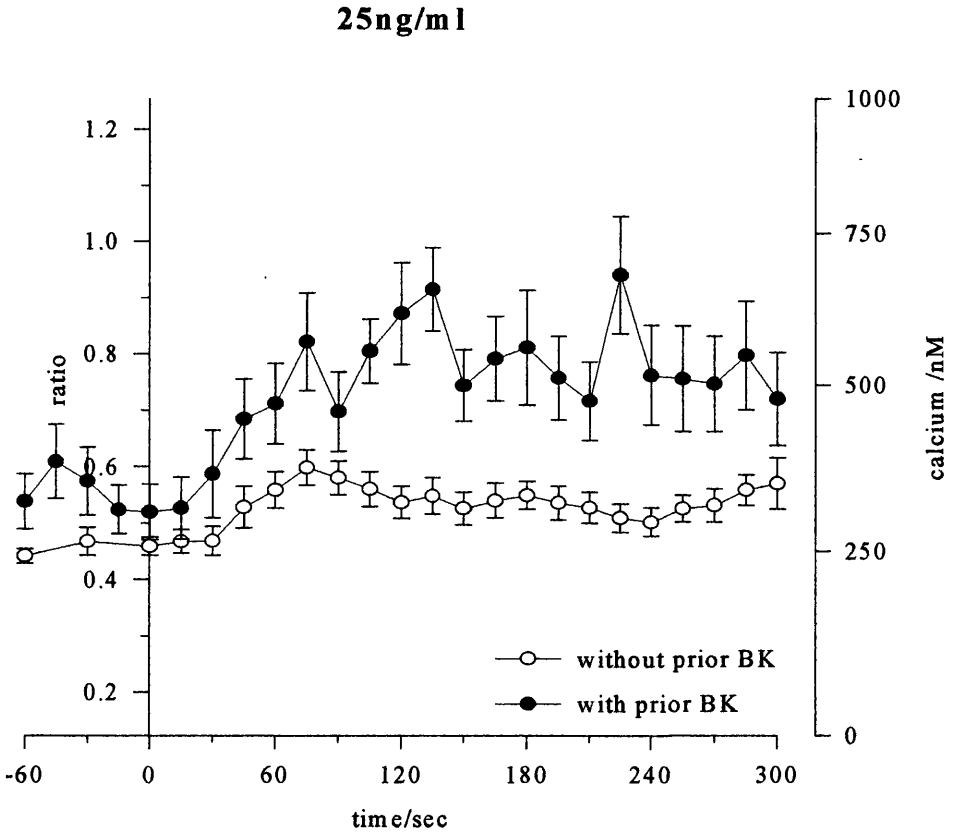
and that individual cells each respond with slightly different rates. The data presented here is consistent with that.

Figure 6.2.2:

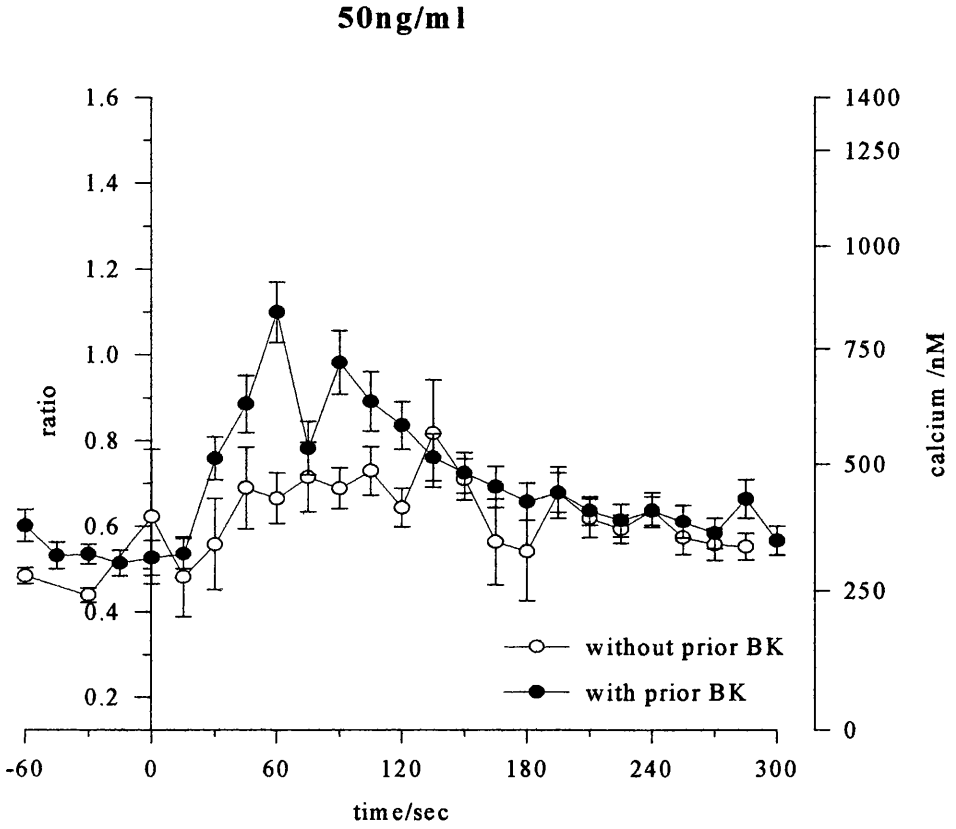
(a)



(b)



(c)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ratio
calcium /nM
time/sec
without prior BK
with prior BK
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1.4
1.2
1.0
0.8
0.6
0.4
0.2
1400
1250
1000
750
500
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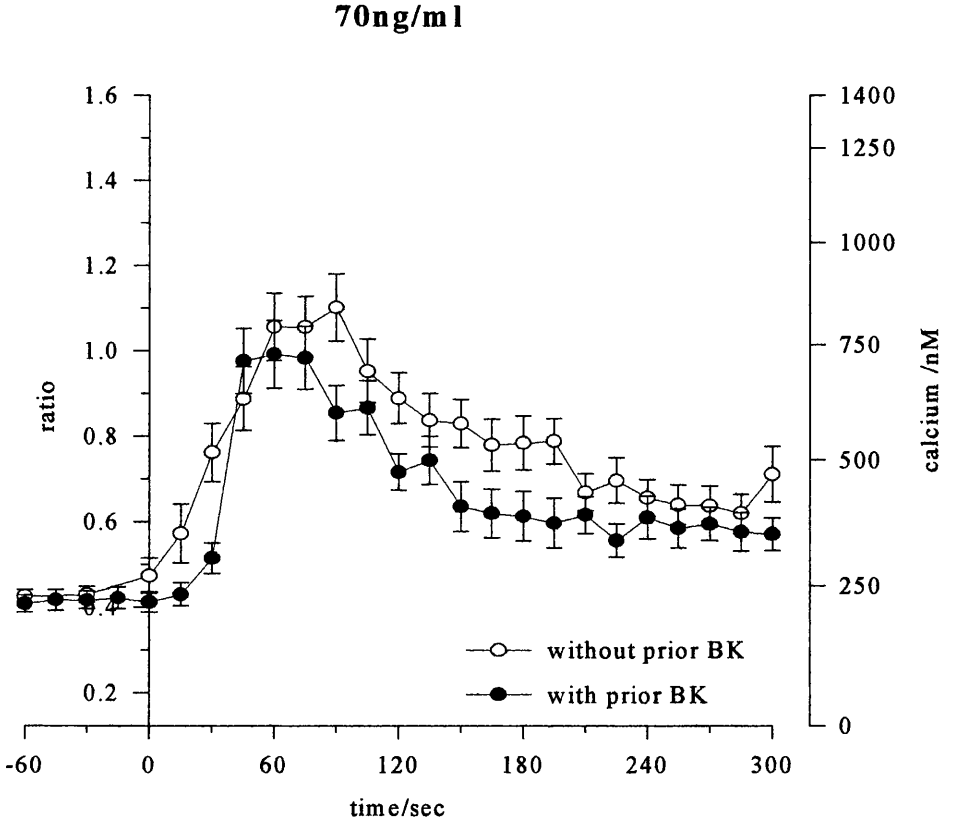
0
-60
0
60
120
180
240
300

(d)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ratio
calcium /nM
time/sec
without prior BK
with prior BK
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1400
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(e)

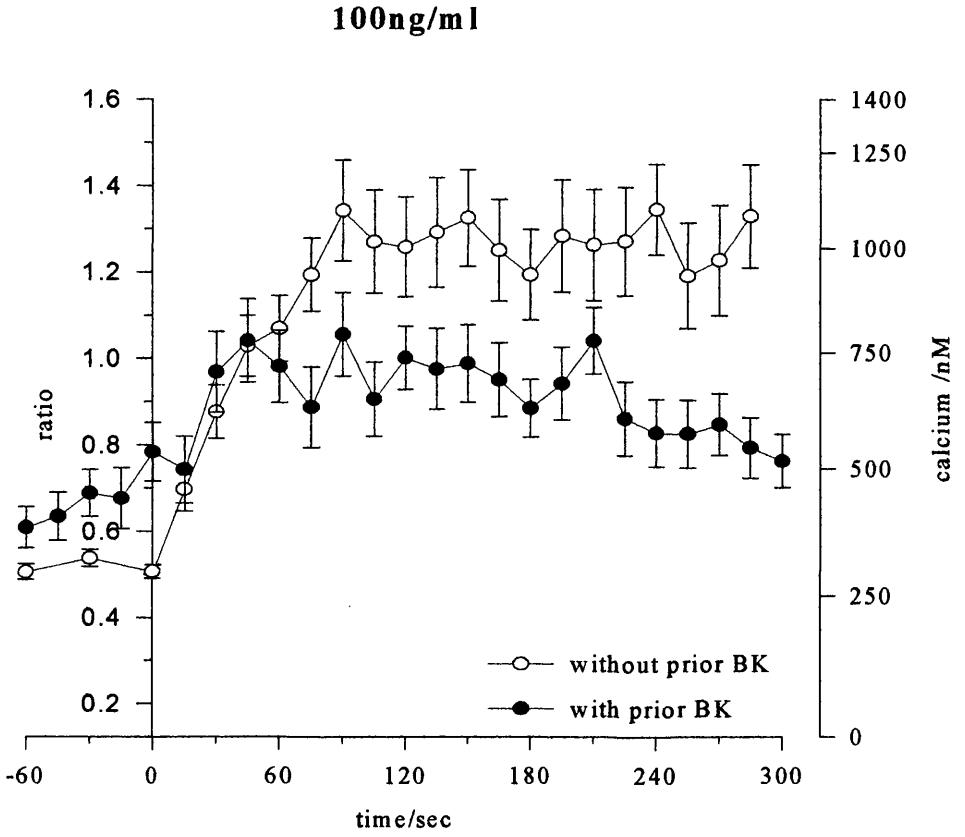

Figure 6.2.2: Time courses for the response by wtA431 cells to rhEGF.

In all cases, open circles indicate no prior treatment, filled circles represent prior treatment with 1μM BK. Each point represents the mean ratio for the time bin (15sec), error bars indicate SEM and the right hand side axis indicates the $[Ca^{2+}]i$ corresponding to the ratio. All data is from a minimum of >3 platings.

(a) Response to 10ng/ml rhEGF. N is 51 (no prior BK) and 50 (with prior BK);

(b) Response to 25ng/ml rhEGF. N is 64 (no prior BK) and 66 (with prior BK);

(c) Response to 50ng/ml rhEGF. N is 58 (no prior BK) and 74 (with prior BK);

(d) Response to 75ng/ml rhEGF. N is 67 (no prior BK) and 71 (with prior BK);

(e) Response to 100ng/ml rhEGF. N is 78 (no prior BK) and 60 (with prior BK);

6.3 Response of wtA431 cells to BK following prior stimulation with rhEGF

Figure 6.3 shows the response to 1μM BK by wtA431 cells, plotted against the concentration of rhEGF used in the pre-treatment. 0ng/ml rhEGF therefore indicates the control response. Cells were stimulated with various concentrations of rhEGF for five minutes, followed by a 60 s washout with HBS, then stimulation with 1μM BK. A single BK concentration point, rather than a range was chosen because the response to BK by A431 cells is monophasic - either the amount of calcium released by exposure to BK will change or it will not, following prior stimulation with rhEGF. Admittedly, the concentration at which a full calcium response would be elicited might be altered, but a switch between different patterns of response is not an option.

Because of the problem with the higher doses of rhEGF having long-lasting calcium changes in the cell, the responses to BK have been expressed as changes in calcium concentration following stimulation, rather than the peak increase attained, since some of the change in calcium is from influx across the cell membrane after the higher doses of rhEGF and not simply as a result of stimulation with BK. The values are plotted as stacked bars, with the lower, open portion of the bar indicating the mean ratio (calcium concentration) immediately before the application of BK. It can be seen that there is a tendency for this ratio to increase as the pre-treatment dose increases. The shaded portion of the bar indicates the rise in ratio (calcium) that followed perfusion with BK. Plotting the mean peak calcium concentration in the cells following stimulation with BK is inappropriate, since, as the pre-treatment doses of rhEGF increase, the

concentration of calcium in the cytoplasm also increases, and, as is evident from figure 6.2.2, the increases remain for a significant period of time. Application of BK was six minutes after the initial treatment with rhEGF each time, and it is clear from figure 6.2.2, that for the doses of 70ng/ml and 100ng/ml, the response of the cells to rhEGF is not over after five minutes and does not appear to be declining either.

There seems to be a stimulatory effect of exposure to rhEGF on the subsequent BK response. With the exception of the 70ng/ml dataset, the hatched portion of the bar, representing the increase of calcium upon BK addition is larger in cells pre-exposed to rhEGF at concentrations 25ng/ml or over. For all prior rhEGF doses from 25 to 100ng/ml, the ratio increase upon subsequent BK application was 0.572 ± 0.046 ($n = 230$). This is significantly larger than the ratio change of naïve cells (0.340 ± 0.036 ($n = 331$); $p < 0.0001$). Thus there seems to be a real potentiation of the response to BK by prior exposure to rhEGF.

In conclusion, these results have shown that:

- prior exposure to BK increases the sensitivity of A431 cells to low doses of rhEGF,
- prior exposure to BK decreases the sensitivity of A431 cells to a maximum dose of rhEGF,
- prior exposure of A431 cells to rhEGF increases the response to BK.

These results will be discussed in the following chapter.

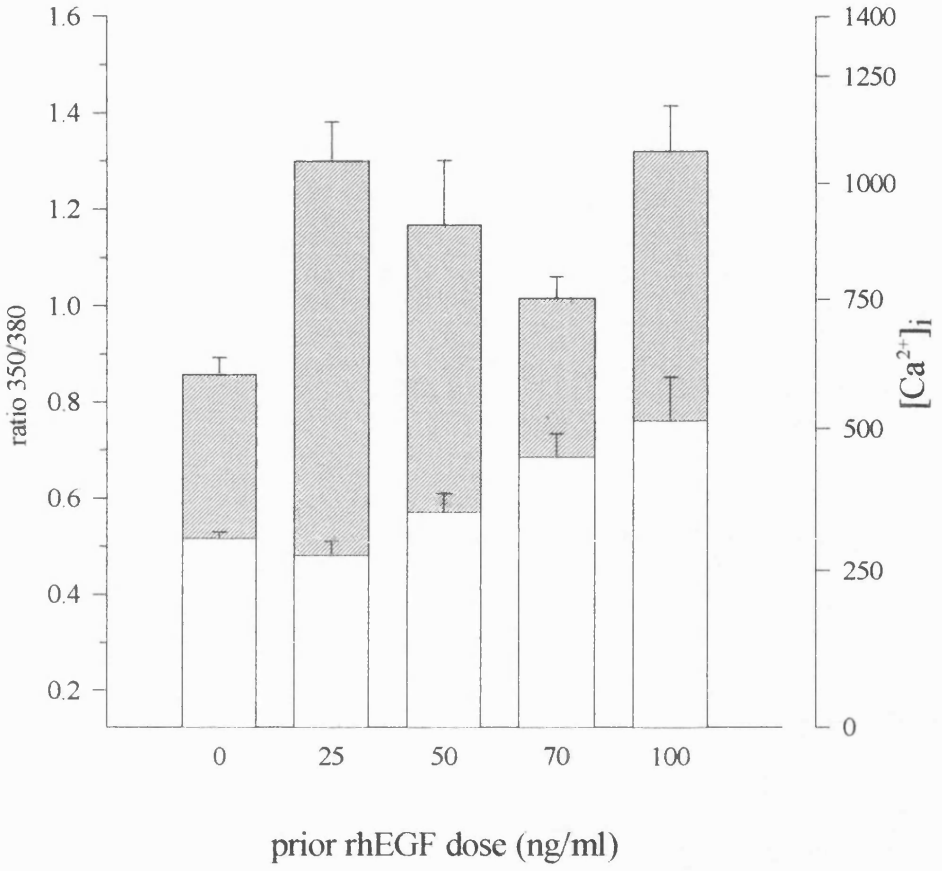

Figure 6.3: Response of wtA431 cells to BK following prior stimulation with rhEGF.

Cells were stimulated with various concentrations of rhEGF for 300 seconds. This was followed by 60 seconds of washout with HBS, then stimulation with 1μM BK. The y axes represent ratio and corresponding $[Ca^{2+}]i$, the figures below the bars indicate the prior dose of rhEGF (0 indicating control). The open portion of the bar indicates the mean ratio ($[Ca^{2+}]i$) immediately preceding BK application. The peak of the shaded bar represents the mean maximum ratio ($[Ca^{2+}]i$) attained following perfusion with BK. Error bars indicate SEM. N are as follows: 348, 51, 67 and 48 (0 to 100 respectively), each from a minimum of 3 platings.

DISCUSSION: AGONIST-INDUCED CALCIUM CHANGES IN A431 CELLS

7.1 CROSS-TALK BETWEEN RECEPTORS

Signalling events at the membrane are not isolated events. Activation of a receptor by its agonist is not always a discrete affair - there may be concomitant activation of another receptor simply because some degree of binding may occur. There are also downstream factors to consider - the activation of a receptor may lead to a cascade of reactions within the cell which could affect membrane receptors other than the one originally activated. Also, the ability of a ligand to promote signalling within the cell may depend on the status of the cell with regards to the cell cycle - it is known that there is cell-cycle dependent regulation of the EGF-R in A431 cells (Newberry and Pike, 1995). This is consistent with the concept that growth factors stimulate cells to undergo growth and division. Once the cell has passed through S-phase, it is committed to cell division. Therefore, it is logical for the sensitivity to growth factors during the phases of the cell cycle during which replication or division of components is occurring, to be reduced in comparison with the sensitivity during G_0/G_1.

This thesis examined the magnitude and pattern of the calcium response to rhEGF following prior stimulation with BK and the magnitude of the calcium response to BK following prior stimulation with rhEGF. The results showed that prior exposure of A431 cells to BK increased the sensitivity of the cells to low doses of rhEGF, but decreased the sensitivity of the cells to a maximum dose of

rhEGF. Also, prior exposure to rhEGF increased the response to BK. I shall discuss these in turn.

7.1.1 Prior exposure to BK increases the sensitivity of cells to low doses of rhEGF

Examining the peak calcium response one can see that when cells have been pre-treated with BK for a 60 second period beginning four minutes before the rhEGF stimulation, there is a shift in the curve to the left. The agonist concentration at which the curve for pre-treated cells peaks is 50ng/ml rhEGF, with the calcium response to 25ng/ml rhEGF also significantly higher than the control values. The response to higher doses of rhEGF are similar to those in naïve cells. It would appear that it has become easier for a lower concentration of agonist to produce the $InsP_3$ peak. I shall discuss the possible reasons for this effect.

Phosphorylation/dephosphorylation of the EGF-R

The fact that the curve has been shifted to the left could indicate that the affinity of the EGF for its ligand has increased and so a lower concentration of ligand produces a greater response. This result is inconsistent with Hosoi's model (Hosoi, Kurihara and Ueha, 1993) which predicts that BK increases the phosphorylation of the EGF-R through the actions of PKC, reducing the binding of EGF to its high affinity site. PKC is calcium dependent - a rise in calcium stimulates its activity. It is also activated by DAG, and so BK, through increasing intracellular concentrations of DAG and calcium would activate PKC and down-regulate the EGF-R through increased phosphorylation. If so, a shift in the dose response curve to the right would be predicted. However, Hosoi and colleagues

also found BK had a second effect - causing dephosphorylation through the activation of a phosphoprotein phosphatase, allowing homeostatic control over other receptor function by regulating the level of phosphorylation. Pre-treatment of the cells with BK has increased the sensitivity of the EGF-R and the most likely explanation of this is that dephosphorylation of the receptor has occurred. However, other explanations could also account for this effect and I will discuss two here.

Receptor activation of PLC

Increased action of phosphatases in the cell could cause de-phosphorylation of the EGF-R and thus inactivation. Conversely, increased phosphorylation of the cytoplasmic domain could lead to increased activity of PLCγ although there is no evidence for either of these processes occurring as the result of cross talk between receptors.

$InsP_3$-R

As discussed in section 1.2.1, the sensitivity of the $InsP_3$-R depends upon the level of calcium within the store, cytosolic calcium concentrations and the phosphorylation state of the receptor. The $InsP_3$-R shows a bell shaped dependence on cytosolic calcium, with the highest open probability at 300nM (Bezprozvanny, Watras and Ehrlich, 1991; discussed by Miyazaki, 1995). It is possible that prior stimulation with BK has increased the sensitivity of the $InsP_3$-R by producing microdomains of high calcium concentration in the vicinity of the receptor. It may also be that increased cytosolic calcium (in response to BK) triggers a number of reactions within the cell that leads to alterations in the sensitivity of the $InsP_3$-R, perhaps through alterations in the phosphorylation state of the receptor.

7.1.2 Prior exposure to BK decreases the sensitivity of the cells to a maximum dose of rhEGF

Although the response to low levels of rhEGF following pre-treatment with BK is increased, the response to the maximum dose is depressed. There are a number of explanations for this effect.

Modulation of the receptor

It is unlikely that modification of the receptor through altered phosphorylation states could cause increased sensitivity to low doses of rhEGF, but inhibition to high doses of rhEGF.

Availability of PIP_2

Since both pathways converge at this point, depletion of the PIP_2 pool in the membrane by prior stimulation of the cell with one agonist, would lead to a reduced calcium concentration in the cell in response to the maximum rhEGF dose through a lowered concentration of $InsP_3$ in comparison with control cells. However, most workers assume that the availability of the substrate is not limiting in cells. Recent work in HL60 cells has shown that sufficient PIP_2 to drive $InsP_3$ production is produced on demand when cells are stimulated (Cunningham *et al*, 1995).

$InsP_3$-R

The sensitivity of the $InsP_3$-R is affected by the state of filling of the internal stores (Nunn and Taylor, 1992). Depletion of these stores would lead to a decrease in $InsP_3$-R sensitivity. The level of store filling is also important in its own right, as well as being an influence on the activity of the $InsP_3$-R. Obviously if the stores are depleted, there is less calcium to be released.

Removal of calcium from the cytosol

The activity of the calcium pump Ca-ATPase is regulated by calmodulin. An increase in the cytosolic free calcium which activates calmodulin will therefore modulate the activity of the Ca-ATPase, increasing its activity and removing free calcium from the cytosol more rapidly. Hence, even if the same amount of calcium is released via the signalling pathways, its increased removal from the cytosol across the membrane or into stores would present as a reduced free calcium concentration. However, it is unlikely that this would only become apparent following exposure to the highest dose of rhEGF and not also be having a visible effect on the response to lower concentrations of rhEGF.

Thus although clear evidence of a mechanism is lacking, the simplest explanation for the reduced response to a maximum dose of rhEGF following BK exposure is that the intracellular calcium stores are somewhat depleted.

7.1.3 Prior exposure to rhEGF increases the response of cells to BK

There is a significant increase in the change in cytosolic calcium evoked by exposure of the cells to BK following prior exposure to rhEGF. I shall discuss the possible explanations of this effect.

Receptor activation of PLC

Stimulation of A431 cells with EGF suppresses BK-activation of the cAMP pathway through tyrosine phosphorylation of $G_{s\alpha}$ although there does not appear to be similar inhibition of G_q mediated inositol phosphate production (Liebmann *et al*, 1996). It is possible that pre-treatment of A431 cells with rhEGF leads to an alteration in the phosphorylation state of the BK-R such that receptor activation of PLC is enhanced, and the level of $InsP_3$ in the cell following exposure to BK is greater.

$InsP_3$-R

As discussed above (section 7.1.1) the sensitivity of the $InsP_3$-R depends upon cytosolic calcium concentrations. When stimulation with BK follows the higher doses of rhEGF, there remains an increased cytosolic free calcium level. Since there is a bell shaped dependency on cytosolic calcium, and the concentration before BK application is close to the peak of this dependency, it is likely that this is resulting in an increase in the open probability of the $InsP_3$-R at the same level of $InsP_3$, thus potentiating the increase in cytosolic calcium concentration following exposure to BK.

Although previous biochemical data have shown interaction between the receptors, it has not been demonstrated before whether the biochemical

alterations would lead to an alteration in cell signalling, and, if so, in what way this would manifest itself. It can be seen that physiological effects of cross-talk between receptors obviously occur, both BK affecting the EGF-R/response and EGF affecting the BK-R/response. This has clear biological significance; in the body both agonists will be present and the responsiveness of cells to EGF is likely to be being modified by the BK that is also present. Potentiation of the receptor/response to low levels of EGF may promote responses and/or cell growth, whereas desensitisation of the receptor/response at high levels of EGF may act as a brake on cell responses and cell growth. Likewise, the potentiation of the BK response by EGF may also cause increased cell responses. Both are probably vital for keeping cell signalling under a tight control.

7.2 ANNEXIN VI$^+$ CELLS

7.2.1 Impaired calcium influx

Preliminary work by other members of the department indicated that wtA431 cells that had been transfected with and were expressing annexin VI have decreased growth rates (increased time to doubling) and a reduced response to the growth agonist rhEGF (Theobald *et al*, 1994). It was also believed that the level of confluency of the cells affected the results obtained. Therefore in all experiments reported here, cells were used only on the first day after subbing, thus reducing any effects due to cell cycle and confluency. Also, only single cells, or groups of two or three cells were used in analysis, to ensure that the majority of the cell surface was exposed to bathing solutions and not in contact with other cells.

Since the annexin family of proteins is characterised by possessing both phospholipid and calcium binding sites, it was postulated that the reduced response to growth hormone and reduced rate of cell division was due to the increased concentration of annexin VI binding the calcium ions, thereby removing them from the pool of free calcium ions whose concentration is measured by the calcium-sensitive fluorescent dyes. Indeed, since increased levels of annexin VI within the cells led to a greatly reduced calcium response following stimulation with rhEGF (figure 5.1.3), it would have been reasonable to suspect that this is simply a reflection of the calcium-buffering capacity of the cell being increased by a raised concentration of a calcium-binding protein. Although the Kd of annexin VI for calcium, 1.2μM, is high, the general increase in calcium observed

in these cells is in the region of 600nM, within the range where annexin VI could be exerting a significant buffering effect.

As shown by the results in chapter three, no significant difference between the buffering capacity of the wtA431 cells and annexin VI expressing cells was observed. Both cell types showed an equivalent increase in free calcium concentration under the same conditions. If the annexin VI was truly buffering calcium, a lower increase in free calcium after flash photolysis of NP-EGTA would have been observed. Since annexin VI is not buffering the calcium, some other explanation for the impaired calcium response had to be sought.

Comparison of the responses to rhEGF of the wtA431, control transfectants (C7 and C8) and clones expressing annexin VI indicated both a shift in the dose response curve and a different pattern of response by the clones expressing the protein. Examining firstly the patterns of response, it can be seen in figure 5.2.2 that, while control clones show a marked elevation of calcium above the baseline for several minutes, the transfected cells do not. Furthermore, it has been shown both previously (Pandiella *et al*, 1987; Peppelenbosch *et al*, 1992) and here, that this maintained elevation is dependent on external calcium. As shown in figure 5.2.2, removal of external calcium abolishes the plateau phase of the response to rhEGF in control cells. The cells expressing annexin VI however, show no elevation in free calcium levels in the cytoplasm following the initial peak. In fact, there is no significant difference between the response observed in calcium-free saline and that observed in normal saline, indicating negligible influx of calcium across the plasma membrane in the cells expressing annexin VI.

To further support this theory, experiments were repeated in saline containing 0.5mM $MnCl_2$ (Mn-HBS). Manganese ions enter the cell through open calcium

channels and bind to the calcium sensitive dye, Fura-2, producing a non-fluorescent product. Hence, monitoring the fluorescence of a cell loaded with Fura-2 at the iso-fluorescent wavelength of the dye provides a means for monitoring influx of Mn^{2+} across the membrane through calcium channels. If annexin VI is preventing these channels from opening, or blocking them (whether open or closed) a lower rate of Fura-2 quenching would be observed in cells expressing annexin VI stimulated with rhEGF, than in wtA431 cells or control transfectants under the same conditions. As shown in figure 5.2.4 and table 5.2.4, this is indeed the case. Annexin VI^+ cells show a significantly lower rate of dye-quench when stimulated with Mn-EGF-HBS than the control transfectants. Quench rates before the application of the agonist, yet still in the presence of Mn^{2+} are comparable however, indicating that the quench of dye by the slow inward trickle of Mn^{2+} at rest are equivalent.

Despite clear evidence that calcium influx is impaired in annexin VI^+ cells, it is not lucid from these experiments where the inhibition of calcium influx occurs. The theory at present is that the influx of calcium across the cell membrane in many cells requires activation of PLA_2, although the mechanism of activation remains as yet unknown (Chow and Powis, 1993; Clark and Dunlop, 1991; Kruger *et al*, 1995; Liu *et al*,1993; Murthy, Kuemmerle and Maklouf, 1995; Peppelenbosch *et al*, 1992; Reddy *et al*, 1995; Rzigalinski, Blackmore and Rosenthal, 1996; Törnquist, Ekokoski and Forss, 1994; Williams, Walsh and Doherty, 1994). Activation of PLA_2 results in the production of arachidonic acid. Subsequent processing of arachidonic acid leads to the formation of eicosanoids and leukotrienes. One particular leukotriene, LC_4, has been demonstrated to activate a calcium channel in the plasma membrane of A431 cells, allowing

microdomains of high calcium concentration to exist just beneath the plasma membrane (Peppelenbosch *et al*, 1992). It is postulated that in the close vicinity there are calcium sensitive potassium channels which open in the high calcium concentrations that would occur close to the calcium channels. Opening of these potassium-channels would lead to the efflux of K^+ ions from the cell, and a resultant hyperpolarisation of the plasma membrane. This hyperpolarisation causes a hyperpolarisation-sensitive calcium channel to open, allowing calcium from the external medium to flood into the cell. It is this calcium influx that is believed to be responsible for the prolonged raised calcium concentration within the cell - the plateau - observed following the peak response in wtA431 cells, and which is absent in the annexin VI^+ cells. From the data presented here, it is not possible to tie down definitely, where annexin VI is interfering with this calcium influx - whether it is at the calcium channel, the activation of PLA_2 or some stage in between. Other work carried out in the department however, has shown that the hyperpolarisation of the membrane observed following stimulation with rhEGF is reduced in A6.1 cells in comparison with both wtA431 cells and C7 control transfectants. However, hyperpolarisation of the cells under whole-cell voltage clamp (from a holding voltage of -30mV to -80mV for 150 seconds) produced similar increases in cytosolic calcium. This data, with the data presented in this thesis imply that annexin VI is exerting its inhibitory effect upstream of EGF-induced membrane hyperpolarisation (personal communication from P. Mobbs, Dept. of Physiology, University College, London). It may be possible that annexin VI is buffering the local "hot spot" of calcium produced by the opening of the LC_4-sensitive calcium sufficiently to inhibit the opening of the K^+ channel (and hence prevent membrane hyperpolarisation) but yet be a small enough buffer that

the experiments examining buffering reported here did not discern the difference. It may also be that annexin VI is acting further "upstream", and affecting the activation of PLA_2.

Other aspects of EGF signalling include the activation of MAP-kinase and induction of c-fos, leading to cell growth and division. Work examining this activation in these cells from the department found that activation is normal in the annexin VI^+ cells, indicating that the role of the protein in growth regulation must lie elsewhere.

7.2.2 Shift in the dose response curve

Turning to address the shift to the right of the dose-response curve shown by the annexin VI expressors, it is not instantly obvious why blocking/inhibiting calcium influx would alter the initial, peak response to rhEGF. This peak is due to the release of internal stores and is independent of extracellular calcium concentrations. It is tempting to suggest that a reduction in calcium influx in the cell, over time, leads to an impairment in the filling of the calcium stores since it is known that growing cells in a reduced calcium media does cause depletion of the stores. The $InsP_3$ receptor is known to be sensitive to the degree of store filling (Newberry and Pike,1995). A low calcium concentration within the store reduces the open probability. Thus reduction of store filling could explain the reduced sensitivity of transfected cells to EGF. It takes a higher cytosolic concentration of $InsP_3$ to elicit a release of store calcium that will produce an equivalent change in cytosolic calcium concentration. Only a certain fraction of total store content can be released by agonist activation of $InsP_3$ production, and that if the stores

become excessively depleted, no appreciable amount of calcium is released. Hence, the cells expressing large amounts of annexin VI do not respond to agonist to the same extent as cells devoid of annexin VI.

It was hoped that a comparison of store levels could be made through the use of thapsigargin (TG). TG locks the internal calcium store channels open, allowing the store contents to be "dumped" into the cytoplasm. A comparison of the amount of calcium liberated in each cell type would provide evidence regarding the size of the store and whether store depletion was occurring in the annexin VI^+ cells. It was especially hoped to examine calcium-store magnitude in the annexin VI^+ clone A6.2 which showed little response to rhEGF even at the maximum level of stimulation - 100ng/ml, since if the $InsP_3$ pathway is intact, the stores certainly do not appear to be. However, the experiments were beset by problems to such an extent that no data could be collected for the A6.2 clone, and little for the other clones.

Cells are regularly screened for annexin VI expression and are found to revert back towards the wild type over time. The A6.2 clone was found to have reverted, and so all data from these experiments had to be discarded. There was not sufficient time to transfect more cells to the same level of expression and to repeat this set of data. Some work had been performed previously on C1 cells (a control transfectant), A6.1 and A6.2 cells (annexin VI^+) and their response to BK. The pattern of the response to BK is not different between the clones (figure 5.2.5b), but the peak response recorded is attenuated in the annexin VI^+ cells. Whereas the control transfectants show a peak calcium of 1140nM ± 240nM, cells expressing annexin VI show a peak calcium of 815nM ± 160nM (A6.2) or 630nM

± 95nM (A6.1) (figure 5.2.5a). The difference is only statistically significant with the A6.1 cells in comparison with the control transfectants, although more data could indicate a real difference for the clone A6.2. These data allow one to rule out the hypothesis that the failure of clone A6.2, the heavy annexin VI expressors, to respond to rhEGF is a result of a massive depletion of intracellular calcium stores.

Summary of the effects of annexin VI expression:

BK stimulates increased $[Ca^{2+}]_i$ within the cell by releasing calcium from internal stores. In the same way that EGF raises intracellular calcium initially, the release is from $InsP_3$-sensitive calcium stores within the cell. In the response to BK there is no subsequent influx of calcium across the plasma membrane. Although both agonists increase the levels of $InsP_3$ within the cell, the enzymes promoting PIP_2 hydrolysis differ between the agonists. Whereas EGF binding to its receptor causes receptor-receptor dimerisation and tyrosine phosphorylation, leading to the activation of PLCγ and hence PIP_2 hydrolysis, BK activates PLCβ indirectly, through a G-protein. As this difference exists in the method of activation, it may be possible to dissect out the level at which the impaired response to rhEGF by annexin VI^+ cells lies. Comparing the responses of cells expressing a medium level of annexin VI (A6.1) and cells heavily expressing annexin VI (A6.2) to rhEGF, and their response to BK may allow one to pin-point the place in the signalling pathway that annexin VI is operating at.

To recap, A6.1 cells show a response to rhEGF that is impaired in two aspects. Firstly, the dose-response curve is shifted to the right - it takes a higher concentration of rhEGF to produce the same peak response of calcium i.e., the

same extent of release of intracellular stores. Therefore it would appear that whatever the effect of annexin VI is, with regards to the release of calcium from internal stores, this effect can be overcome by increased agonist concentration. Secondly, the influx of calcium across the plasma membrane - the secondary phase of the calcium response to rhEGF is drastically reduced in the cells expressing annexin VI. This has been demonstrated in both the degree of quenching of Fura-2 by manganese in the presence of rhEGF, and also the pattern of response in Ca-free saline when compared to normal saline.

The response of the A6.1 clone to BK is also impaired. It is significantly lower than the response of control transfectants. This peak calcium response is also a reflection of the release of calcium from intracellular stores. No dose-response curve to BK was performed on either control cells or, so it is impossible to say whether this reduced response is again merely a reflection of a shift in the dose-response curve to the right.

Examining now the response to rhEGF and BK by the clone A6.2, one sees a different pattern of response. These cells do not appear to respond to rhEGF even at high doses - the peak calcium response at 100ng/ml rhEGF is not significantly higher than the resting calcium concentration before agonist application. However, their response to BK does not follow the expected pattern. One would expect these cells to show no response to BK either, but, although the response is impaired, it is significantly above resting values, and indicates these cells <u>can</u> release calcium from intracellular stores.

These differences between the clones may allow one to suggest how annexin VI is operating. Figure 7.2.2(a) shows a cartoon of the signalling pathways for rhEGF and BK.

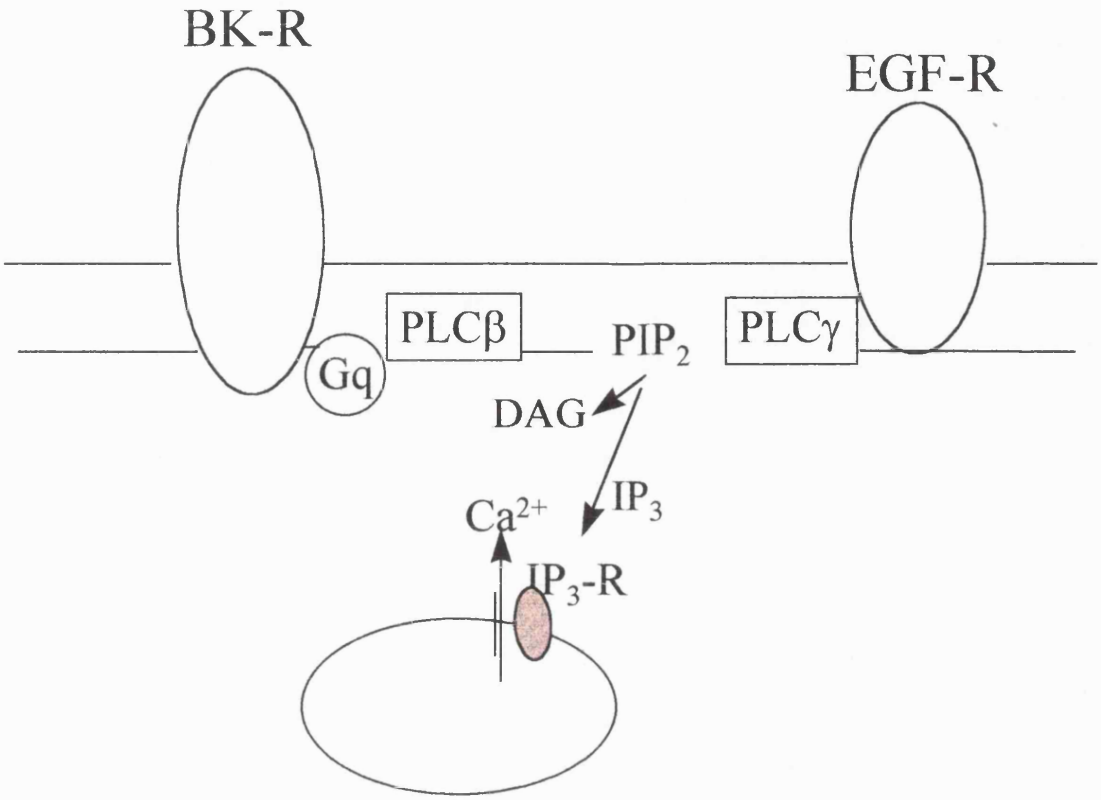

Figure 7.2.2: Cartoon of the pathways involved in $InsP_3$ production in cells.

Regarding only the primary phase of the response to rhEGF, it can be seen that once PIP_2 is hydrolysed, the pathways are common between rhEGF and BK. There are a number of levels at which annexin VI may be exerting its effect - influencing or affecting the receptors, influencing or affecting the levels of enzyme or substrate in the membrane, affecting the hydrolysis of PIP_2, affecting the interaction of $InsP_3$ with its receptor, affecting calcium store levels or buffering the released calcium. I shall discuss each point in turn:

1) Buffering the released calcium

If only the primary response to rhEGF is regarded, it might be thought that the effect of annexin was merely a buffer - that if the cells are stimulated enough, enough calcium would be released to overcome the buffering effect, in cells

expressing annexin VI at a medium level, but, if the cells are swamped with annexin VI, then all responses would be masked. Experiments discussed earlier indicate that there is no difference in buffering between the control cells and the cells expressing annexin VI. As mentioned above, it may be that annexin VI is acting as a calcium buffer at the plasma membrane and inhibiting the calcium-sensitive hyperpolarisation of the membrane. This would not explain the differences observed between the response to rhEGF and the response to BK by the heavily expressing cells. Therefore, the theory that annexin VI is acting merely as a calcium buffer can be discarded.

2) Reducing internal calcium store levels

Again, examining only the responses to rhEGF, it could be thought that the lack of influx across the plasma membrane resulted in a decrease in the intracellular store levels such that greater stimulation was required in A6.1 cells, and that the stores were so impaired in the A6.2 cells that even the maximum dose barely increased intracellular calcium concentrations. However, when the responses of the cells to BK are examined, this possibility is not sufficient to explain the results entirely. As expected, the A6.1 cells showed a reduced increase in calcium on stimulation with BK, compared to control cells. However, if the stores were so run down in the A6.2 cells that $InsP_3$ could not release significant amounts of calcium, one would predict that BK would elicit no response either, but it does. Indeed the response is still lower than that of the control cells, but it is still a response. So, although the stores may be impaired, as expected from reduced influx, there is still some calcium available for release. Indeed, since the size of the stores could not be assessed directly, through TG, there is no firm evidence upon which to base the theory that the stores are impaired. A reduction of the influx via one pathway

does not remove other pathways allowing influx across the cell membrane. Also, if annexin VI is causing the stores to be run down, it would follow that the more annexin VI that is present in the cell the greater the effect and this is clearly not the case for the BK results.

3) Affecting the interaction of $InsP_3$ with its receptor

The same arguments raised in the above section on store levels can be applied here. If annexin VI is interfering with the $InsP_3$-R, the more annexin VI present, the greater the level of interference. Yet again, the results of the A6.2 cells stimulated with BK reduce the weight of this argument significantly. If annexin VI was interfering so much with the $InsP_3$-R to prevent release of internal calcium as a response to rhEGF, it should surely have the same effect in the response to BK. Since A6.2 cells do respond to BK, annexin VI cannot be affecting $InsP_3$-Rs. Also, other studies by S.E. Moss, Dept. of Physiology, University College, London (personal communication) have shown that annexin VI is predominantly localised to the cell membrane, rather than the $InsP_3$-R.

4) Levels of enzyme and substrate in the membrane

These two aspects can be dealt as one, since the same arguments apply. If annexin VI was affecting the levels of PIP_2 (or the extent of hydrolysis) then the BK results would be expected to follow the pattern of the rhEGF results. Since they do not, one can assume that the levels of enzyme and substrate and extent of hydrolysis are unaffected.

5) Receptor-PLC activation

All other possibilities have been ruled out, and in fact, this is the only level at which the two pathways differ, so therefore the only point that could be affected to give different results. From the results discussed here, it is unclear whether

annexin VI is interfering with the EGF-R itself, or the subsequent activation of PLCγ. It cannot be an non-specific inhibitor of PLCs because of the differences in the A6.2 responses to rhEGF and BK.

It is possible that part of the response to BK may be through a non-specific action of BK on the EGF-R. Hence, the majority of the production of $InsP_3$ following stimulation with BK is via activation of PLCβ, but a component part may also be through activation of PLCγ. If annexin VI is interfering predominantly with the activation of PLCγ, this component part of the response to BK will be impaired in both of the annexin VI^+ clones, but unaffected in the cells that do not express annexin VI (the control transfectants). If even a low expression of annexin VI in A431 cells is sufficient to prevent the slight activity of PLCγ by non-specific action, then the impairment of this component of the response to BK by the transfected cells will not be dependent on the level of annexin VI expression and therefore will be equal in both annexin VI^+ clones. In this scenario, PLCβ would be unaffected by annexin VI, but the component of the response due to concomitant EGF-R activation would be lost. Hence, the response to BK would not be significantly different between annexin VI^+ cells, but significantly different from control cells. The results presented here indicate that the response to BK by the annexin VI^+ clones is reduced (significantly for the A6.1 clone) and that there is no significant difference between the responses of the annexin VI^+ clones to BK. It would appear that annexin VI is affecting either the EGF-R or the activation of PLCγ.

Combining these results with earlier work performed on CHO cells lines with a mutated EGF-R (Clark and Dunlop, 1991) and other work on the annexin VI^+

cells within this department, the evidence seems to indicate that annexin VI is interfering with the cytoplasmic domain of the EGF-R. Clark and Dunlop examined the role of the cytoplasmic sub-domain of the EGF-R and found that it was essential for PLA_2 activation via EGF. Other work in this department indicates that the link between EGF-R activation and calcium influx is impaired at the level of reduced hyperpolarisation as a response to rhEGF in annexin VI^+ cells. Figure 1.2.4 is a cartoon of the pathway envisaged linking EGF-R activation and calcium influx. Activation of EGF-R causes the activation of PLA_2, possibly through tyrosine phosphorylation. There follows a sequence of reactions by which arachidonic acid is converted to leukotriene C_4 (LC_4). LC_4 opens a calcium channel, allowing the influx of calcium. This is not itself the secondary calcium response, but produces localised "hot spots" of calcium under the plasma membrane. The "hot spots" then cause the activation of a calcium sensitive potassium channel, which opens, allowing potassium to leave the cell, with a resultant hyperpolarisation of the membrane. The hyperpolarisation activates a voltage sensitive calcium channel. It is the opening of this channel that is the cause of the secondary calcium increase in the cell (Peppelenbosch *et al*, 1992). Patch clamp studies from other members of this department indicate that hyperpolarisation in the absence of rhEGF causes equal calcium changes in A6.1 cells and C7 cells. However, rhEGF causes a smaller hyperpolarisation in the A6.1 cells than in the C7 cells (personal communication from Dr. S. Bolsover and Dr. P. Mobbs, both of Dept. of Physiology, University College, London).

If annexin VI is affecting the kinase activity of the EGF-R, and PLA_2 is activated through tyrosine phosphorylation, then the action of annexin VI seems obvious - it interferes with the phosphorylation of PLCγ to a large extent in

heavily expressing cells, but to a lesser extent in the A6.1 cells, such that it can be overcome by increased levels of agonist. Annexin VI also interferes with the phosphorylation/activation of PLA_2 such that, ultimately, LC_4 is not produced to the same extent as in the control cells, and so there is reduced hyperpolarisation in annexin VI^+ cells with the result of impaired calcium influx across the cell membrane.

Since a component of the BK response could stem from EGF-R activation, at a level low enough not to induce calcium influx across the membrane (the pattern of response to EGF changes with increasing dose, from a sharp peak, to a peak-plateau - Cheyette and Gross, 1991), the loss of this component in annexin VI^+ cells would account for the impaired responses to BK, but yet allow some response still to be evident, even in the heavily expressing cells. It would also account for there being no significant difference between the responses elicited between the two annexin VI^+ cells types.

7.2.3 Clone reversion

The problem of clone reversion must be considered in any further work. The freezing down and re-thawing of clones applies a huge selection pressure to the cells. Those cells that cope well with the freeze-thaw cycle and recover the best will tend to influence and monopolise the character of the clone culture. Annexin VI^+ transfectants show a reduced growth rate compared to parentals. In the continuous cultures, which were maintained relatively confluent, this does not present a problem. However, when a frozen aliquot of cells was thawed and grown up to re-establish a culture, there was a strong tendency for those few

revertants within the original frozen group to divide faster and hence come to dominate the culture. For this reason, it proved all but impossible to store annexin VI transfected cells as frozen aliquots and it is now the policy of the Moss laboratory (Dept. of Physiology, University College, London) to create new transfectants as required. In the present study, not only were cells screened regularly to check annexin VI expression, but the response to rhEGF was monitored throughout the course of these experiments.

7.2.4 Expression during cell cycle

Recent work (Francia *et al*, 1996), has shown that there are few changes in the spectrum of proteins expressed by a tumour cell, when its character changes from benign to malignant. One of these changes is the loss of expression of annexin VI. It seems probable, in the light of my research, that in "normal", non-malignant cells, annexin VI is acting as a brake on cell division and growth, by altering the amount of calcium that can enter a cell following stimulation with a growth hormone. If this brake is lost, the cell is able to decrease doubling-time. Expression of other annexins alters with cell cycle (Chiang, Schneiderman and Vishwanatha, 1993). There is as yet no evidence that the expression of annexin VI alters with cell cycle, but responses to rhEGF certainly differ depending on the number of days that have passed since the cells were subbed. All experiments in this study were conducted within a set window of time after subbing (16-30 hours) and it had been observed during preliminary work (personal communication from Dr. P. Mobbs, Dept. of Physiology, University College, London) that responses of the annexin VI^+ clones did vary with time since plating,

suggesting that annexin VI expression or effectiveness may alter with time. Further experiments are needed to confirm this suspicion. It must be considered that many cells grow and divide perfectly adequately, even with an equivalent level of annexin VI expression as the clones examined here.

To summarise, annexin VI is unlikely to be acting as a calcium buffer, nor interfering with signalling pathways downstream of PIP_2 hydrolysis. Instead, it would appear, in A431 cells to interfere with PLCγ activation in some way, probably at the level of the EGF-R. This would explain both the shift in the dose-response curve and the significant response of A6.2 cells to BK. If the protein is acting at the receptor level, it would be necessary for a higher concentration of agonist to be present to produce a comparative activation of the receptor leading to an increased calcium concentration in the cell, consistent with Cheyette and Gross (1991). The small amount of EGF-R activation that could occur with BK stimulation is also lost in both annexin VI^+ clones, hence there is some reduction in the peak $[Ca^{2+}]_i$ in annexin VI^+ cells in comparison with the parental cells. The responses of the annexin VI^+ cells are not significantly different from each other. Obviously cells manage to grow and divide, despite the presence of annexin VI. Expression of the protein may alter with position in the cell cycle - exerting a stronger effect in some periods of the cell cycle than during others.

CONCLUSIONS

This thesis examined the capacity of a neuronal cell line to buffer calcium, the cross-talk between different receptors and the resultant effect on the calcium responses to two agonists, and the effects on calcium signalling of expression of the protein annexin VI.

The calcium buffering capacity of N1E-115 cells, a neuronal cell line was assessed through the use of a caged-calcium compound, NP-EGTA. Photo-destruction of NP-EGTA led to a small release of calcium, distributed uniformly throughout the cytoplasm of the cells. The calcium release was small enough that no calcium buffers within the cell would saturate giving misleading results, as may occur at the periphery of a cell when the calcium load applied to a cell is via an influx of calcium across the plasma membrane. The result obtained indicated a very high buffering capacity in these cells. This may reflect the technique used to assess the buffering capacity in these cells, or may simply reflect a high calcium buffering capacity in these cells.

The ability of a cell to control any calcium changes that occur is vital. A concentration of free calcium in the cytoplasm that remains high for significant periods of time can be pathological for cells. Since calcium is such an important second messenger within cells, tight control over intracellular concentration must be maintained through controlling the magnitude or duration of calcium changes, or both. The remainder of this thesis examined calcium signalling in A431 cells, in two aspects. Firstly the interplay between agonists on the calcium responses by cells was examined, by comparing both the magnitude of calcium changes observed following application of two agonists, and also the pattern of the

calcium change observed. Secondly, the role of the calcium sensitive phospholipid binding protein, annexin VI, in calcium signalling was examined.

Cells are not isolated units, that are only stimulated by one agonist at a time, and the interplay between agonists and both their specific receptor and other receptors is crucial. In this thesis, the cross-talk between BK and EGF on A431 cells was examined. The response of the cells to EGF following prior stimulation with BK was compared to the response with no prior stimulation. Pre-treatment with BK shifted the dose response curve to EGF to the left, but also depressed the peak response. It is likely that the EGF-R has been affected through some downstream effect of BK binding to the BK-R.

When cells were stimulated with EGF followed by BK, the response to BK was increased. Pre-treatment with EGF, which increased the magnitude of the response to BK, led to potentiation of the BK-R or a downstream component.

Expression of annexin VI in A431 cells is known to reduce growth rates and induce contact-inhibition. The role of annexin VI in the calcium signalling elicited by stimulation of the cells with EGF was examined, as well as a comparison of the changes in cytosolic calcium concentration observed following photodestruction of NP-EGTA with parental cells, lacking in annexin VI. The results of this thesis show that annexin VI is affecting the response of cells to EGF by inhibiting calcium influx across the plasma membrane - the secondary calcium response to EGF. It may be doing so by buffering local calcium levels at the membrane, leading to the inhibition of hyperpolarisation, but the comparison of calcium release between parental cells and annexin VI^+ cells indicated no difference in the ability to buffer calcium. Other experiments comparing the response of the cell types to BK indicated an effect on calcium signalling being

located to the EGF-R. It would seem more likely that annexin VI is exerting its effect at the level of the EGF-R than by buffering calcium.

Calcium is an important, near ubiquitous second messenger in cells. Anything that affects the magnitude, pattern or duration of calcium changes in a cell will have profound effects on the response of the cell to a stimulus. This thesis examined three factors that could affect the calcium signals within a cell - the ability of a cell to buffer calcium changes, thus affecting the magnitude of a calcium change observed for a given calcium load; the cross-talk between different receptors, which affects both the magnitude and pattern of response to an agonist; and the effect of specific protein expression, which again modulates both the size and style of response seen in cells expressing the protein in comparison with cells which are lacking in the protein. It is evident from this study that a huge amount of variation in the responses of cells can be elicited by altering any or all of these factors.

ACKNOWLEDGEMENTS

I would like to express my thanks to Dr. Steve Bolsover in whose laboratory this research was completed and for his help and encouragement throughout; Dr. Steve Moss, and Dr. Pete Mobbs, again, for help and encouragement and Katie Patel and Anne Palmer for their assistance in cell culture. I also wish to thank my family, and all the friends and colleagues whose unwavering support and belief in me have been invaluable.

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