Manipulation of second messengers by ectopic expression of receptors in transgenic *Drosophila*

A thesis submitted for the degree of
Doctor of Philosophy at the University of Glasgow

Martin Kerr

Division of Molecular Genetics
Institute of Biomedical and Life Sciences
University of Glasgow
Glasgow
G11 6NU
UK

The research reported in this thesis is my own work except where otherwise stated, and has not been submitted for any other degree.

Martin Kerr

Abstract

The combination of physiology and genetics affords opportunities to perform experiments with greater precision, and with less risk of artefact, than heretofore possible. This is illustrated here by demonstrating the selective manipulation of the second messengers cyclic GMP (cGMP), cyclic AMP (cAMP) and calcium (Ca^{2+}) in the renal (Malpighian) tubule of the genetic model organism, *Drosophila melanogaster*. Pharmacological intervention typically involves bathing a whole tissue in a drug, or the use of agents with broad specificities that might confound interpretation. However, ectopic expression of receptors in a cell renders it sensitive to the putative ligand, and –provided that the necessary internal machinery is present- gives it the potential to respond in defined ways. In the case of *Drosophila*, it is possible to target such transgenes with great precision, using the GAL4/UAS 'binary' enhancer trap system, allowing the targetting of defined cells in an organotypic context.

The importance of the second messengers cGMP, cAMP and Ca^{2+} in control of fluid transport makes the Malpighian tubule an ideal testbed for such technology. The actions of known hormones can be compared with the results obtained by manipulating levels of the second messenger through which these are thought to act.

Flies were constructed or obtained transgenic for the rat atrial natriuretic peptide receptor, GC-A, the *Drosophila* $5HT_{7Dro}$ receptor, and the *Drosophila* $5HT_{1ADro}$ receptor, under control of the UAS or heat-shock promoter. Tubules dissected from such flies were then demonstrated to show diuresis induced by application of rANP or 5HT, whereas controls showed no response. Second messenger measurements showed that the rat GC-A receptor acted to raise cGMP levels, the $5HT_{7Dro}$ receptor to raise cAMP levels, and the $5HT_{1ADro}$ receptor to raise intracellular calcium. In addition, modulation of both cell-

specific cyclic nucleotide phosphodiesterase activity and cell-specific cyclic nucleotide-dependent kinase activity of tubules with elevated cyclic nucleotide levels was observed, implicating these enzymes as key regulators/effectors of signalling in tubules. Cross-talk between cGMP, cAMP and Ca^{2+} was also assessed and shown to be variable, depending on the particular cell type in which the signal was generated. This reveals further complexity in the control of tubules and suggests a distinct role for cyclic nuleotide-gated channels in principal cells.

This study has validated ectopic transgene expression as a generic technology with potential beyond Malpighian tubules: in principle, such transgenes can be expressed specifically in any population of cells that can be delineated by a GAL4 driver line.

Abbreviations

AEBSF	4-(2-aminoethyl) benzenesulphonyl fluoride hydrochloride
ANP	atrial natriuretic peptide
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BDGP	Berkeley *Drosophila* Genome Project
bp	base pairs
BSA	bovine serum albumin
Ca^{2+}	Calcium ions
CAP_{2b}	cardioacceleratory peptide 2b
cAK	cAMP-dependent kinase
cAMP	3′-5′ cyclic adenosine monophosphate
cDNA	complementary DNA
cGK	cGMP-dependent kinase
cGMP	3′-5′ cyclic guanosine monophosphate
cNMP	3′-5′ cyclic (nucleotide) monophosphate
CNG	cyclic nucleotide gated channel
CNS	central nervous system
cpm	counts per minute
dATP	2′ deoxyadenosine triphosphate
dCTP	2′ deoxycytosine triphosphate
dGTP	2′ deoxyguanosine triphosphate
dNTP	2′ deoxy(nucleotide) triphosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetra acetic acid (disodium salt)
ER	endoplasmic reticulum
EtBr	ethidium bromide
fmol	femtomoles

g	gram
GC-A	guanylate cyclase receptor A
GDP	guanosine diphosphate
GFP	green fluorescent protein
GPCR	G protein-coupled receptor
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
h	hour
IBMX	3-isobutyl-1-methylxanthine
I.D.	inner diameter
$InsP_3$	inositol 1,4,5-trisphosphate
kb	kilobases
kDa	kiloDaltons
M	molar
mol	moles
max.	maximum
mg	milligram
ml	millilitre
mm	millimeter
mM	millimolar
min	minute
mRNA	messenger RNA
ng	nanogram
nM	nanomolar
nmol	nanomoles
NO	nitric oxide
NOS	nitric oxide synthase
O.D.	outer diameter

Ore R	Oregon R
PBS	phospate buffered saline
PCR	polymerase chain reaction
PDE	phosphodiesterase
PKC	protein kinase C
PLC	phospholipase C
pmol	picomoles
PMSF	phenylmethylsulphonylfluoride
PtdIns(4,5)P_2	phosphatidyl inositol bisphosphate
RIA	radioimmunoassay
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per min
RT-PCR	reverse transcriptase polymerase chain reaction
sec	second
SDS	sodium dodecyl sulphate
sGC	soluble guanylate cyclase
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
u	unit
UAS	upstream activating sequence
UV	ultraviolet light
V-ATPase	vacuolar H^+ adenosine triphosphatase
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
µg	microgram
µl	microlitre
µM	micromolar
°C	degrees Celsius
5HT	5-hydroxytryptamine (serotonin)

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Chapter 1

Introduction

1.1 *Drosophila melanogaster* as a model organism

In biology, an extremely useful way to elucidate gene, cell and tissue function is to take a transgenic approach. However, the more biomedically relevant the chosen model, the more difficult it becomes to manipulate genetically. Therefore, a balance has to be met whereby analogy to higher organisms can be drawn, yet genetic manipulation can be achieved easily and effectively. *Drosophila melanogaster* lends itself as an excellent model organism with an intermediate level of genomic complexity, yet a sophisticated genetic technology as a result of a century of analysis. This has led to a comprehensive bed of literature, biological techniques and genetic information being freely available to the *Drosophila* community. Furthermore, *Drosophila* have a short generation time and are easy to culture in hundreds/thousands of distinct genetic lines, cheaply, and in a limited space. The completion of the *Drosophila* genome project means that the entire euchromatic genome has been sequenced, and the task of disrupting every gene is well underway. This has been made possible by P element mutagenesis, discussed in section 1.2.

1.2 P elements

1.2.1 P element structure

The manipulation of P elements revolutionised *Drosophila* genetics. They are used as tools in a vast array of genetic, developmental and cell biological studies. The P element is a 2.9kb autonomous transposable element found in natural populations of *Drosophila melanogaster*, with the ability to hop from one chromsomal location to another throughout the genome. They were first identified as the cause of a syndrome called P-M hybrid dysgenesis in progeny of certain crosses of *Drosophila* strains. This brings about a number of disadvantageous traits, such as male recombination, the generation of mutations, chromsomal aberrations and sterility. P-M dysgenesis occurs when

males of a paternally contributing (P) strain are mated with females of a maternally contributing (M) strain, but not when the reciprocal cross is performed. P strains contain multiple P elements dispersed all over the major chromsome arms, whereas M strains lack P elements. The P elements are repressed in the P strains, regulated by the P cytotype, but when placed in the cytoplasmic background of the M cytotype which lack repressor they become derepressed, and thus transpose at high rates (Engels and Preston, 1979).

Transposition is made possible by the P element encoding a *trans*-acting 87kD transposase from four long open reading frames (Rio *et al*, 1986). P elements also contain 5′ and 3′ perfect 31 bp terminal inverted repeats and an 11 bp subterminal inverted repeat needed in *cis* for efficient transposition (O'Hare and Rubin 1983; Mullins *et al*, 1989). P element transposition is genetically regulated, with activity of the transposase restricted to the germline as the result of differential splicing (Laski *et al*, 1986). In the major P element RNA transcript, found in somatic cells, the first three open reading frames are joined by the removal of two introns. However, the removal of a third intron, containing an in frame stop codon, is necessary for transposase production. This intron is only removed in the germline (Figure 1.1). Importantly, however, P elements with internal deletions that remove or disrupt the transposase gene are still capable of transposition when supplied with an exogenous source of transposase (Robertson *et al*, 1988).

The sites of P element integration in the genome are highly variable, but there is a strong preference for euchromatin (the non-repetitive regions of chromsomes) and the 5′ untranslated regions of genes (Spradling *et al*, 1995).

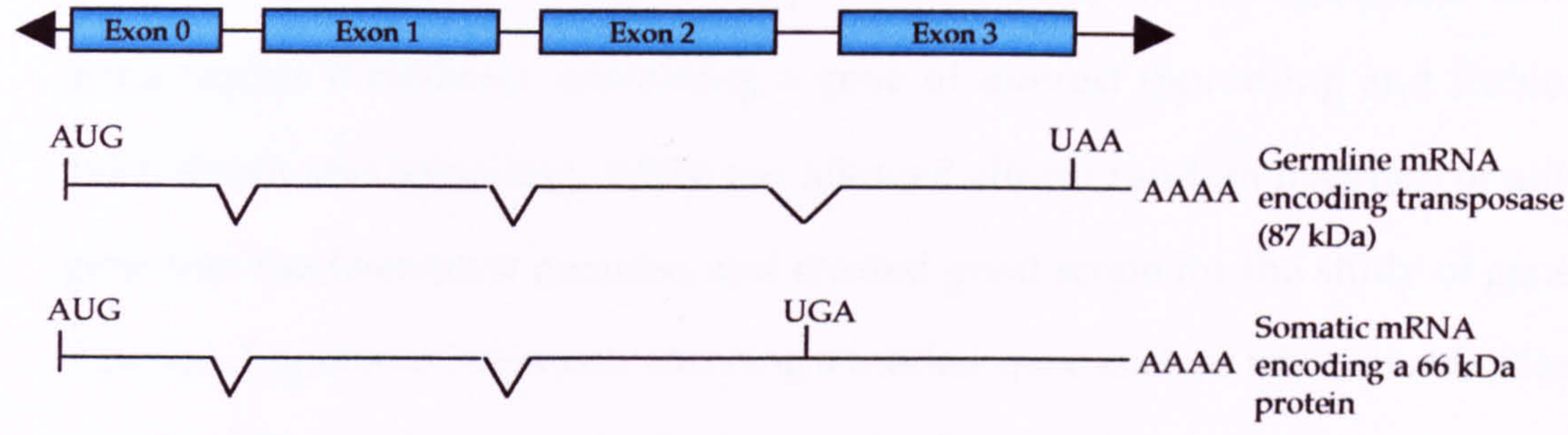

Figure 1.1: Structure of P elements

The full length 2.9 kb P element has four long ORFs separated by introns. The P element contains perfect 31 bp terminal inverted repeats (arrowheads). Germline transcripts, spliced as shown, provide functional transposase. Somatic transcripts, which retain the intron between exons 2 and 3, encode a prematurely truncated and thus non-functional transposase. Internally deleted P elements do not provide functional transposase and are thus non-autonomous, but they retain *cis*-acting determinants that allow their mobilisation in the presence of exogenous transposase. Δ2-3 elements, from which the third intron has been removed by *in vitro* manipulation produce transposase in both germline and somatic tissues.

[Adapted from Sentry and Kaiser, 1992].

1.2.2 Germline transformation

Germline transformation of *Drosophila* M strains with engineered non-autonomous P elements containing a gene of interest (Spradling and Rubin, 1982; Rubin and Spradling, 1982), has allowed almost random insertion of any gene into the *Drosophila* genome, and created great scope for the study of gene function. Engineered elements carrying a marker gene such as *white*, enable flies with insertions to be recognised when strains lacking the marker gene are used for transformation. Exogenous transposase can be supplied in a number of ways to these non-autonomous elements. The P element vector can be coinjected with purified protein (Kaufman and Rio, 1991), or with a separate vector containing the transposase gene that has the third intron removed (named Δ2-3 helper plasmids) (Figure 1.2). Similarly, the P element construct can be injected into embryos of a transgenic strain with constitutively active transposase, such as P[ry^{+} Δ2-3] (99B) flies (Laski *et al*, 1986; Robertson *et al*, 1988) and then the transposase element can be removed by outcrossing to avoid further transposition. P element constucts are injected into the posterior end of embryos at the transition between syncitium and cellular blastoderm. The DNA injected into the region of pole cell formation can become internalised and transpose into germline chromsomes. This is not frequent on a per cell basis, but nonetheless provides acceptable transformation efficiencies. Newly integrated P element constructs in the germ cells are stably inherited by the progeny of transformed individuals, and fly lines can then often be bred to homozygosity for the insertion.

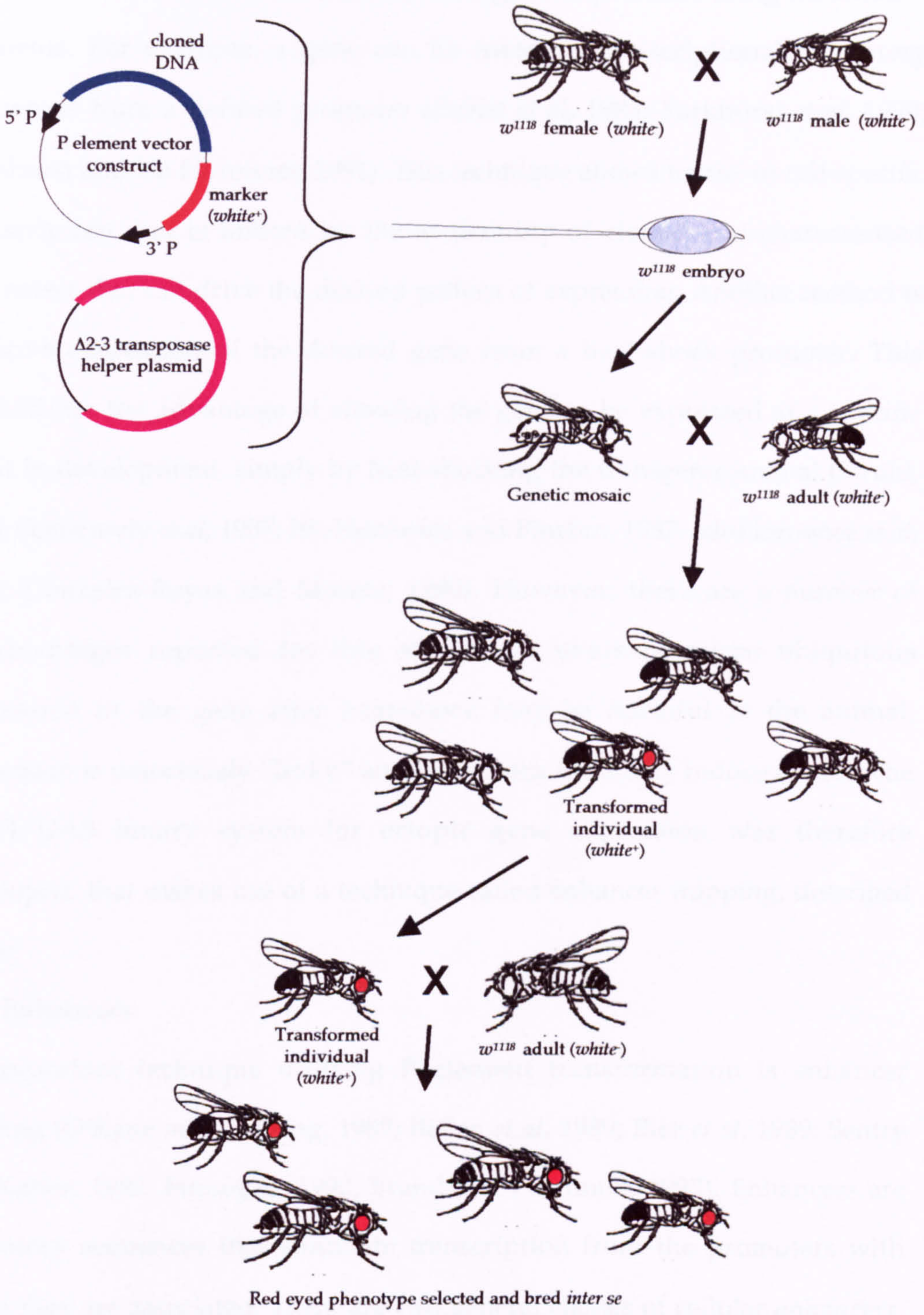

Figure 1.2: Germline transformation of *Drosophila*

Embryos from the strain w^{1118} were coinjected with a non-autonomous P element vector (containing the gene of choice and the *miniwhite* marker gene), and Δ2-3 helper plasmid, which encodes an 87 kD transposase. Surviving adults were crossed with w^{1118} and progeny were screened for insertion of the P element, indicated by expression of the marker gene *miniwhite* producing a red eye phenotype. Transformed individuals were backcrossed with w^{1118} and bred *inter se*, to establish independent transgenic lines.

[Adapted from Guo, 1996].

There are several methods to manipulate gene expression using modified P elements. For example, a gene can be fused to transcriptional regulatory sequences from a defined promoter (Zuker *et al*, 1988; Parkhurst *et al*, 1990; Parkhurst and Ish Horowicz, 1991). This technique allows tissue- or cell-specific transcription, but is limited by the availability of cloned and characterised promoters that can drive the desired pattern of expression. Another method is to drive expression of the desired gene from a heat-shock promoter. This method has the advantage of allowing the gene to be expressed at a specific point in development, simply by heat-shocking the transgenic animal (Struhl, 1985; Schneuwly *et al*, 1987; Ish-Horowicz and Pinchin, 1987; Ish-Horowicz *et al*, 1989; Gonzalez-Reyes and Morata, 1990). However, there are a number of disadvantages reported for this method of overexpression; ubiquitous expression of the gene after heat-shock may be harmful to the animal, expression is notoriously "leaky" and heat-shock itself may induce effects. The GAL4/UAS binary system for ectopic gene expression was therefore developed, that makes use of a technique called enhancer trapping, described below.

1.2.3 Enhancers

An important technique utilising P element transformation is enhancer trapping (O'Kane and Gehring, 1987; Bellen *et al*, 1989; Bier *et al*, 1989; Sentry and Kaiser, 1992; Finnegan, 1992; Brand and Perrimon, 1993). Enhancers are regulatory sequences that stimulate transcription from the promoters with which they are associated. There are two general classes of cellular enhancers, namely inducible enhancers and temporal and tissue-specific enhancers. They are a major mechanism for regulating gene expression in eukaryotes. Once activated by binding of a specific protein, they act as sites for the assembly of transcription initiation complexes. Enhancers differ from other regulatory sequences in several ways. Firstly, they may be upstream or downstream of the

gene(s) and several kb away from the gene(s) that they control. Secondly, they can act in either orientation and so can simultaneously influence expression of two genes, one on each side of the enhancer sequence. Thirdly, they can be on the opposite strand of DNA as the regulated gene (although they must be on the same DNA molecule), and lastly, they are not gene specific, but tissue specific and will preferentially enhance transcription from the nearest promoter (Smith-Keary, 1991).

1.2.4 First generation enhancer traps

Enhancer trap vectors were designed containing modified P elements with the transposase gene deleted, into which a reporter gene cloned downstream of a minimal promoter region (insufficient in itself to induce transcription) could be inserted. This P element could be introduced into the genome by germline transformation with an exogenous source of transposase, as described earlier. The reporter gene would then fall under the regulation of the nearest enhancer to the P element in the genome. Initial enhancer traps used the *lacZ* gene, which encodes β-galactosidase from *Escherichia coli*, as the reporter gene (O'Kane and Gehring, 1987) (Figure 1.3A). These P{*lacZ*} lines expressed *lacZ* in a manner reflecting that of the endogenous gene that the enhancer controls. Enhancer activity was then visualised by staining for β-galactosidase activity with the chromgenic substrate X-gal. This P{*lacZ*} fusion was an efficient tool for the isolation and characterisation of genes and their enhancers, simply by providing knowledge of their pattern of expression (rather than screening for phenotypes caused by mutations), and also for the generation of a wide variety of cell type-specific markers. For example, Perrimon *et al* (1991) generated flies containing *lacZ* fused to either the weak promoter of the P element *transposase* gene, or to partial promoters from the *even-skipped*, *fushi-turazu*, or *engrailed* genes. These lines were used to follow specific cell lineages in development and also to analyse the phenotype of several developmental mutants. Similarly,

Gonczy *et al* (1992) used marker lines to label early stages of male but not female germ cell differentiation, which is useful in the analysis of germline sex-determination.

1.2.5 Second generation enhancer traps

A second generation enhancer trap was developed by Brand and Perrimon (1993), named P{GAL4} using the yeast transcription factor GAL4 as a reporter instead of β-galactosidase. GAL4 is functional in *Drosophila*, but does not activate transcription of any endogenous genes (Fischer *et al*, 1988). It can therefore be used to direct expression of other transgenes placed under the control of the GAL4-dependent promoter UAS_G (*U*pstream *A*ctivating *S*equence for galactose). Constructs containing the UAS_G sites upstream of the *hsp70* promoter, which lacks its upstream regulatory sequence, may be used to generate flies that contain a gene of choice that will remain silent until crossed with a GAL4-expressing fly (Brand and Perrimon, 1993). This binary system, in which expression of the gene under control of UAS_G requires the presence of GAL4, means that these lines can be maintained as separate transgenic stocks (Figure 1.3B). This system has the benefit of allowing the experimenter to study phenotypic consequences of misexpression (which could include lethality) in the progeny of a simple cross, rather than trying to maintain (over extended periods of time) lines that could potentially be very sick. Furthermore, with a large number of P{GAL4} lines available, virtually any expression pattern of the gene under control of UAS_G can be achieved, either spatially or temporally.

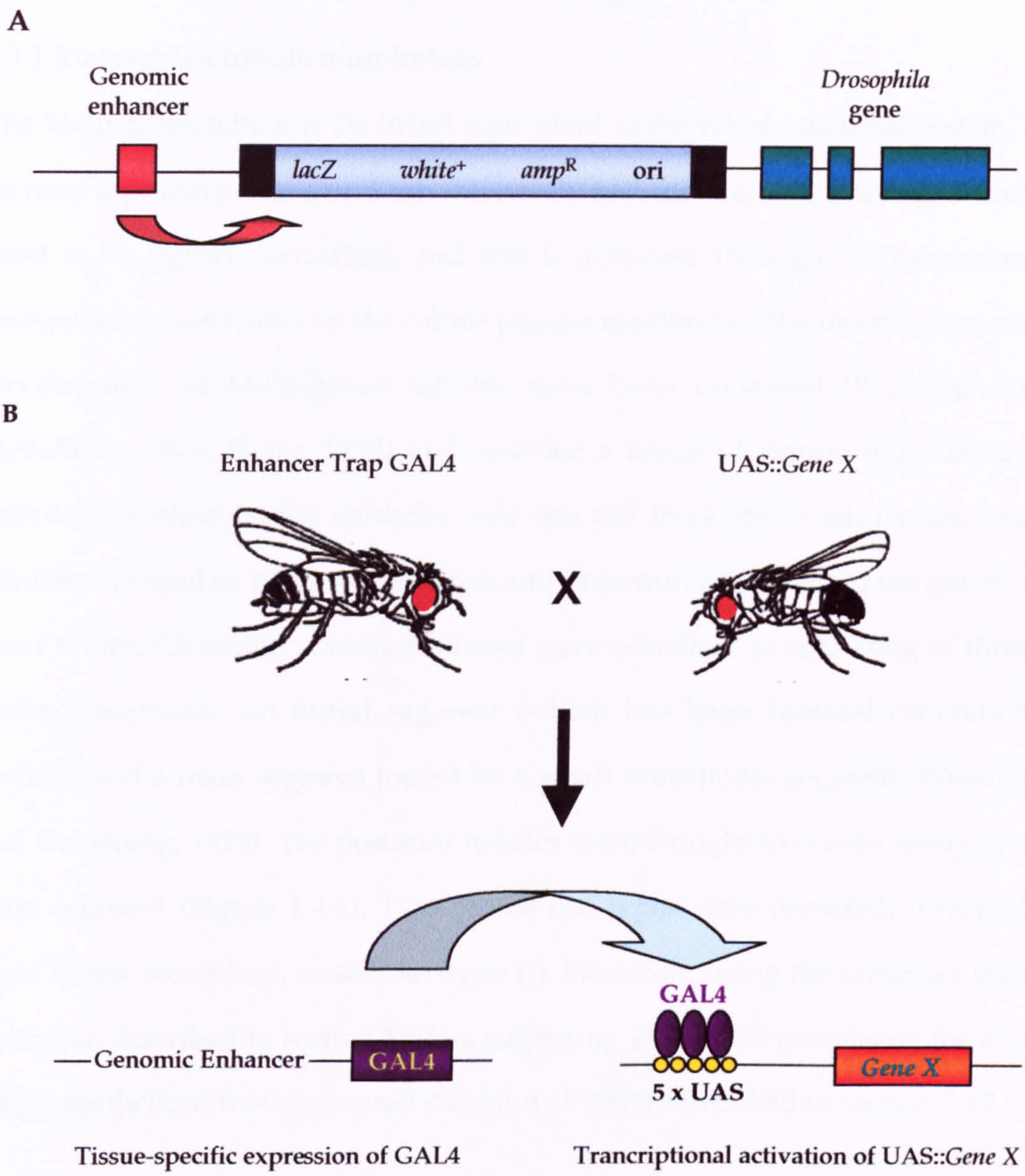

Figure 1.3: Enhancer trap gene expression

A: A first generation enhancer trap element inserted within a *Drosophila* gene. The pattern and timing of expression of the reporter, *lacZ*, is dependent upon the specific genomic context in which it is integrated. *white*$^{+}$ is a marker that confers red eye colour in a *white*$^{-}$ genetic background, and thus allows flies containing new insertions to be recognised. The ampicillin resistance determinant (*amp*^{R}) and *E. coli* origin of replication (ori) to facilitate plasmid rescue of flanking sequences.

[Taken from Sentry *et al*, 1994].

B: A GAL4 enhancer trap element. The pattern and timing of GAL4 expression is similarly context dependent, and can be used to drive expression of a secondary reporter gene linked to the GAL4-responsive promoter, UAS_G. This binary system separates the target gene from the driver in two distinct transgenic lines.

[Adapted from Brand and Perrimon, 1993].

1.3 The *Drosophila melanogaster* Malpighian tubule

1.3.1 Malpighian tubule morphology

The Malpighian tubule is the insect equivalent to the vertebrate renal system. It secretes a primary urine, and can selectively reabsorb solutes. These processes need to be tightly controlled, and this is achieved through neurohormone recognition by receptors on the tubule plasma membrane. The morphology and development of Malpighian tubules have been reviewed (Wessing and Eichelberg, 1978; Skaer, 1993) and describe a tissue of simple morphology. Tubules are blind-ended epithelia only one cell thick, and each fly has four tubules arranged as two pairs, anterior and posterior, connected to the gut by a short ureter. Classically, anterior tubules were described as consisting of three distinct segments, an initial segment (which has large luminal concretion bodies), and a main segment joined by a small transitional segment (Wessing and Eichelberg, 1978). The posterior tubules were thought to consist solely of a main segment (Figure 1.4A). Two tubule cell types were reported: principal (type I) and secondary, or stellate (type II). However, using the enhancer trap technique described in section 1.2.5, a surprising amount of complexity for this simple epithelium was uncovered (Sözen *et al*, 1997), described in section 1.3.2.

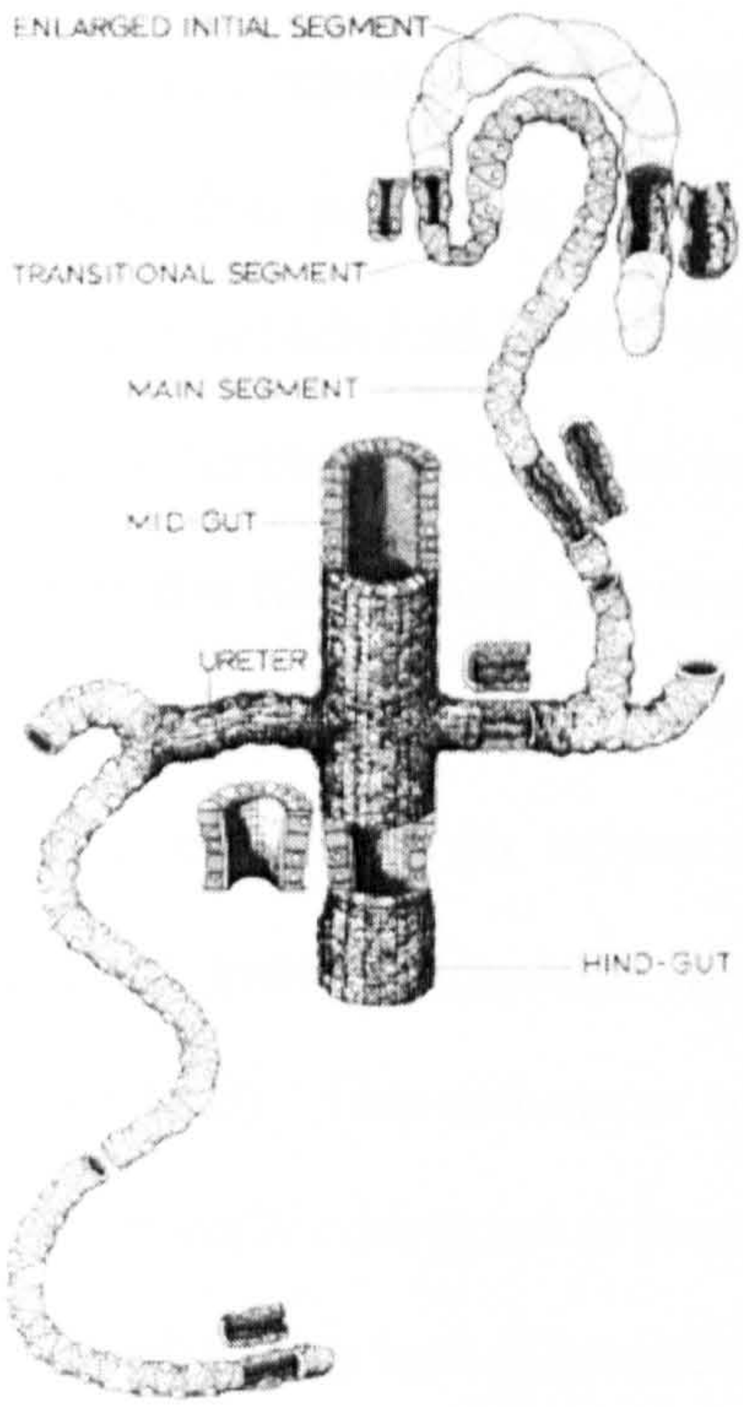

Figure 1.4: Morphology of the Malpighian tubule

A: The classical morphology of the Malpighian tubules, illustrating main, initial and transitional segments of the anterior tubule(s) and one long main segment of the posterior tubule(s). Both anterior and posterior pairs of tubules are attached as pairs to the gut by a common ureter.

[Taken from Wessing and Eichelberg, 1978].

B: Summary of tubule regional architecture, as revealed by P{GAL4} enhancer trap lines. The different subregions of the tubule are indicated and the numbers of principal and stellate cells in each region are shown.

[Taken from Sözen *et al*, 1997].

1.3.2 Malpighian tubule enhancer traps

A panel of over 700 P{GAL4} enhancer trap lines were analysed (Sözen *et al*, 1997) of which approximately 10% revealed interesting expression patterns in Malpighian tubules of the progeny of crosses with UAS_G::*lacZ*. This analysis revealed that posterior tubules did in fact have small initial and transitional segments, which had been overlooked in other studies. Also, the main segment could be further subdivided in both anterior and posterior tubules. The lower third of the tubule and the ureter were also shown to be genetically separable from the main segment, which could itself be resolved into three subregions: a lower tubule and an upper and lower ureter. Furthermore, these newly identified genetic domains were organised in number to single-cell precision (Figure 1.4B). This enhancer trap analysis also revealed further cell subtypes: principal cells comprise at least two distinct subpopulations, bar-shaped cells are found in the initial and transitional segments of the anterior tubules, and "tiny" neuroendocrine cells were revealed in the lower tubule and ureter. These tiny cells are speculated to secrete hormones involved in the control of fluid secretion and reabsorption by tubules. This study gave important insight into how the organism views its tubule structure, rather than how the experimentor had previously viewed it. Furthermore, P{GAL4} lines that revealed interesting patterns of expression were established as homozygous stocks and can now be used to drive expression of any reporter under UAS control in specific cells or regions of tubules.

1.3.3 A model for epithelial fluid transport and second messenger signalling

Classically, insect Malpighian tubules have been used as models of epithelial fluid secretion and its neurohormonal control (Maddrell, 1981; Maddrell, 1991; Maddrell and O'Donnell, 1992). Malpighian tubules from *Drosophila melanogaster* were demonstrated to be amenable to physiological study, with the development of an assay for measurement of fluid secretion (Dow *et al*, 1994b) (Figure 1.5). However, tubules from *Drosophila melanogaster* are suited not only to classical physiology, but also molecular genetic analysis, thus allowing an approach termed integrative physiology to be deployed (Dow and Davies, 2001). Taking this approach, we can address cell function in an organotypic context.

Intracellular second messengers are important modulators of epithelial function. An integration of signals leads to overall function and tight control of second messenger levels, both within and between cells, is needed. Receptors expressed on the plasma membrane are involved in control of second messenger levels, by recognising and binding ligands, and then relaying that signal through alteration of second messenger levels. Heterologous expression of a receptor in a cell line similar to the cell type from which it was isolated helps characterise its function, but may not reflect any subtle differences apparent in an *in vivo* multicellular environment. The aim of this project is to distinguish the signalling pathways that coordinate fluid transport, using ectopic expression of receptor transgenes in tubules that signal *via* distinct second messengers, and to characterise the pharmacology of these receptors in a differentiated multicellular environment.

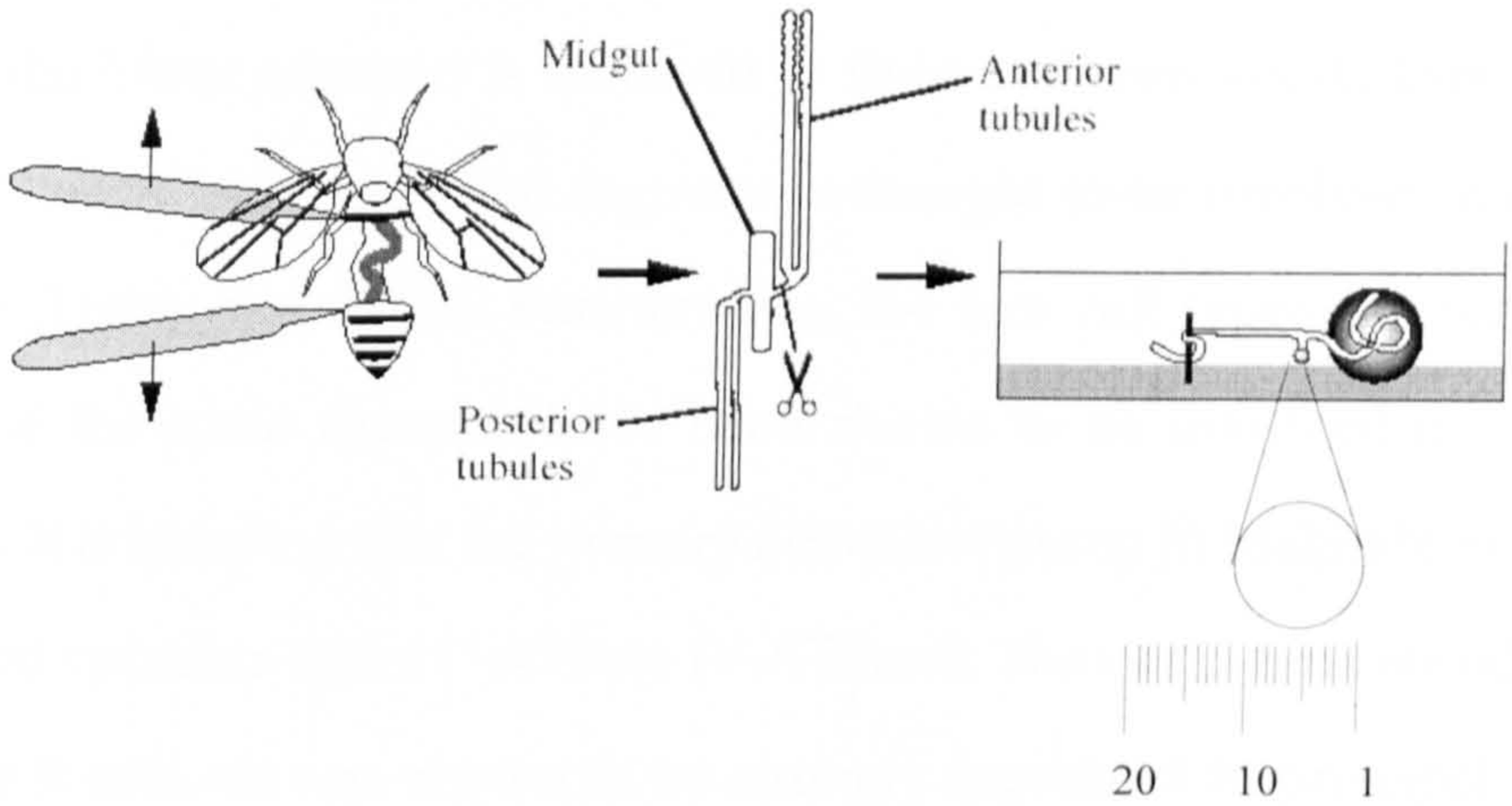

Figure 1.5: Measurement of fluid secretion by *Drosophila* Malpighian tubules

Malpighian tubules were dissected from the fly in Schneider's insect culture medium (Gibco BRL) in pairs, by severance of the ureter. One tubule was wrapped around an anchoring pin whist the other remained in a 9 µl drop of 1:1 mixture of Schneider's: *Drosophila* saline, positioned with a depression in a paraffin wax base. Fluid secretion is measured under mineral oil to prevent evaporation. Secreted fluid forms as a spherical drop at the ureter, and can be removed at intervals with a fine glass rod. The diameter of the drop can be measured with an ocular micrometer, allowing volume of secreted fluid to be calculated.

[Taken from Dow *et al*, 1994b].

1.3.4 Distinct functions of tubule regions and cell types

The regions of the tubule have different functions and physiological properties. It has been demonstrated that the main segment performs a secretory role, whereas the lower segment is involved in fluid reabsorption (O'Donnell and Maddrell, 1995), and the initial segment is thought to be involved in calcium transport (Dube *et al*, 2000). Furthermore, the two cell types (principal and stellate) of the main segment have been shown to be involved in separate functions. It is accepted that the primary active ion pump in Malpighian tubules is an apical vacuolar-type H^+-ATPase (V-ATPase). The *vha55* gene encoding the V-ATPase B-subunit was shown to be strongly expressed in principal cells of the main segment of tubules by enhancer trap analysis (Davies *et al*, 1996), indicating that the V-ATPase was confined to these cells. The V-ATPase maintains a proton gradient across the apical membrane that drives movement of alkali cations from cell to lumen through apical sodium/proton (Na^+/H^+) and/or potassium/proton (K^+/H^+) exchangers (antiporters). The V-ATPase also establishes a favourable electrical gradient for the flow of anions, such as chloride (Cl^-), from cell to lumen. This in turn establishes an osmotic gradient that promotes the movement of water from cell to lumen as a secondary consequence of ion transport. Microelectrode measurements of transepithelial potentials in tubules showed that stimulation of tubules with cyclic nucleotides made the lumen more electropositive through the activation of the apical electrogenic V-ATPase in principal cells (O'Donnell *et al*, 1995). This response could also be achieved by treating tubules with the neuropeptide CAP_{2b} (cardioacceleratory peptide 2b, a neuropeptide peptide isolated from *Manduca sexta* related to the *Drosophila melanogaster capa* peptide family), which acts to raise intracellular cGMP in principal cells of tubules through activation of a nitric oxide synthase (NOS) sensitive soluble guanylate cyclase (O'Donnell *et al*, 1998; Davies *et al*, 1995; Davies et *al*, 1997). However, changes in extracellular

chloride levels did not alter this potential, suggesting that chloride must pass through cells by another route (O'Donnell *et al,* 1996). It was subsequently demonstrated by vibrating probe (or self-referencing electrode) analysis (which measures tiny disturbances in the electric field around a tissue) that chloride movement was confined to a small number of current density hot-spots. These hot-spots were consistent with the positions of stellate cells, and could be abolished with low-chloride saline or by incubating tubules with chloride channel blockers. The chloride permeability of tubules can be raised by the insect neuropeptide leucokinin, which has been shown to raise intracellular calcium levels rapidly and specifically in stellate cells (O'Donnell *et al*, 1998; Rosay *et al*, 1997). This is conclusive evidence that anion and cation transport is not only functionally separable, but controlled by different cell types of tubules. This is summarised in Figure 1.6.

Much insight has been gained about the signal transduction pathways that control fluid transport in tubules; however an approach whereby the actions of a single second messenger could be isolated and its downstream effectors subsequently analysed may be able to identify points of crosstalk. It is perhaps important to define what is meant by crosstalk at this point. In a particular cell, if signal components from one pathway can influence components of another pathway, then crosstalk is said to occur. However the definition can also be extended to "cross activation", whereby actions at common targets or actions at separate targets ultimately produce an identical action on a single effector.

In order for a specific second messenger signal transduction pathway to be successfully dissected out, receptors and their ligands have to be carefully chosen. This study would not be easy using the endogenous neuropeptides at our disposal (CAP_{2b}, capa peptides, CRF-like peptide and leucokinin). Although the main second messengers activated have been identified, there is no way of determining whether these peptides are only being bound by receptors on one

particular cell type. There is also ambiguity as to the action of other components in these pathways. For example, when CAP_{2b} or a CAPA peptide binds to its receptor, there is an initial rise in intracellular free calcium concentration ($[Ca^{2+}]_i$) which is necessary for the activation of NOS (Rosay *et al*, 1997; Davies *et al*, 1997; Kean *et al*, 2002). Therefore, the activation of soluble guanylate cyclase by NO to produce cGMP is much further down the signal cascade than calcium (Ca^{2+}). Intracellular Ca^{2+} is known to have fundamental roles in control of fluid secretion independent of cGMP, making the role of cGMP difficult to unmask in this instance.

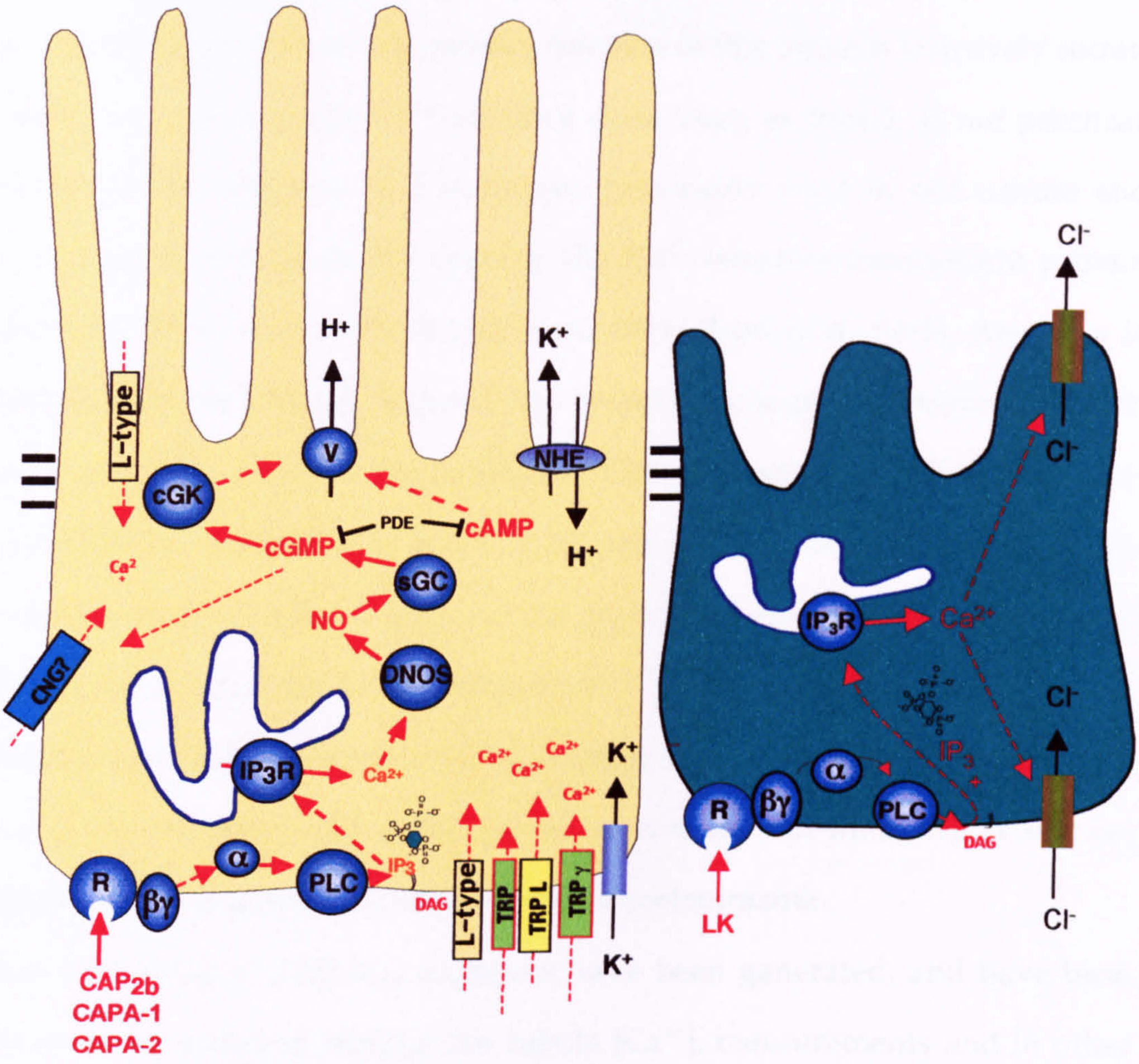

Figure 1.6: Summary of cell signalling pathways in the cells of the Malpighian tubule

The neuropeptides CAP_{2b} and members of the CAPA family bind to a receptor on the basolateral membrane of principal cells and stimulates a rise in $[Ca^{2+}]_i$ (through a G protein-coupled receptor). This rise activates DNOS, producing NO, which activates soluble guanylate cyclase to produce cGMP. The rise in cGMP results in increased activity of the V-ATPase and an increase in fluid transport. Increased V-ATPase activity and the subsequent increase in fluid transport can also be achieved when tubules are treated with cAMP. The neuropeptide Drosokinin binds to a G protein-coupled receptor on the basolateral membrane of stellate cells and stimulates a rise in $[Ca^{2+}]_i$ exclusively in the stellate cells, which increases chloride shunt conductance. This, in turn, increases fluid transport.

[Taken from Dow and Davies, 2001].

1.3.5 Measuring intracellular calcium concentration in *Drosophila* tubules

Malpighian tubules are too small to measure intracellular calcium concentrations ($[Ca^{2+}]_i$) with traditional techniques, such as using ion-specific microelectrodes. Also, as the primary function of this tissue is to actively secrete waste, using fluorescent Ca^{2+}-sensitive dyes, such as fura-2, is not practical. However, the adaption of a technique previously used in cell culture and plants, is to transgenically express the Ca^{2+}-sensitive luminescent protein aequorin (Sheu *et al*, 1993; Knight *et al*, 1991; Brini *et al*, 1995). Aequorin is isolated from the jellyfish *Aequoria victoria* and is a complex of aequorin and the substrate coelentrazine. In the presence of Ca^{2+}, aequorin is rapidly converted to apoaequorin, coelentramide and CO_2 (Figure 1.7). This reaction results in the emission of blue light, which can be quantified into $[Ca^{2+}]_i$. Therefore, if apoaequorin is placed under UAS control, it can be expressed in a cell-specific manner using any of the panel of GAL4 driver lines available. Thus, cell-specific $[Ca^{2+}]_i$ can be measured non-invasively, and in real-time, when tubules expressing the transgene are incubated with coelentrazine.

Flies containing a UAS::aeq transgene have been generated, and have been shown to be useful in Malpighian tubule $[Ca^{2+}]_i$ measurements and in other tissues (Rosay *et al*, 1997; Rosay *et al*, 2001). Cell-specific Ca^{2+} signals can now be resolved in tubules in an organotypic context, probably with less artefact than other techniques, and has aided the characterisation of the CAP_{2b}/capa and Drosokinin signal transduction pathways discussed previously (Rosay *et al*, 1997; Terhzaz *et al*, 1999; O'Donnell *et al*, 1998; MacPherson *et al*, 2001; Kean *et al*, 2002). Expression of transgenic receptors in the same cell type that aequorin is expressed in will therefore allow the evaluation of cross-talk between cyclic nucleotide and Ca^{2+} signalling.

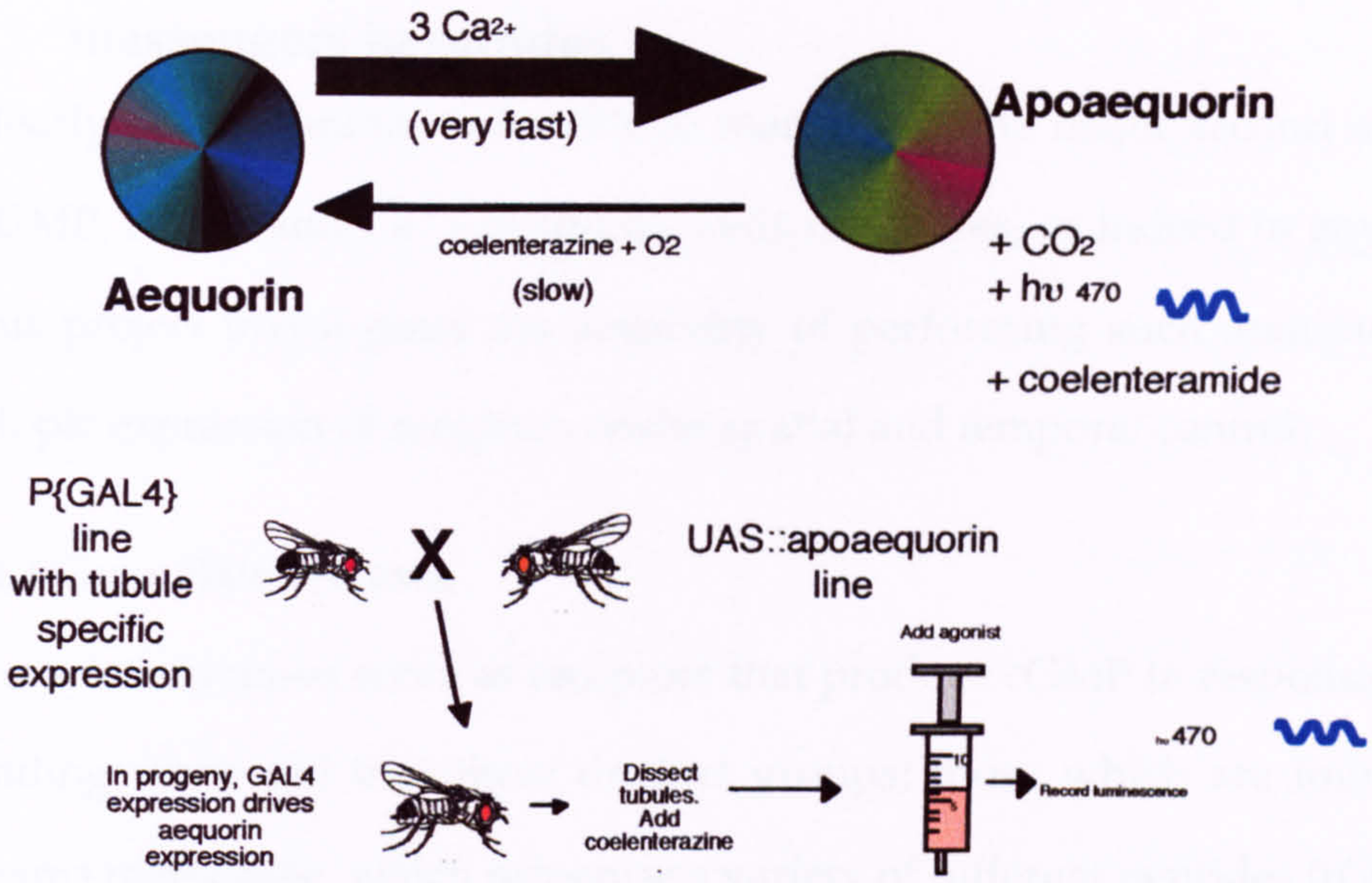

Figure 1.7: Aequorin expression in the Malpighian tubule.

Progeny of a cross between flies that express GAL4 in a cell-specific manner and flies containing the apoaequorin gene under the control of UAS express the apoaequorin gene in a cell-specific manner. The constitution of apoaequorin with coelentrazine, results in the slow formation of aequorin, which, in the presence of calcium, rapidly converts back to apoaequorin. This results in the emission of blue light, which is quantifiable, allowing calcium concentration within the cells to be calculated at any given time.

[Adapted from Terhzaz *et al*, 1999].

1.4 Receptors as tools for specific manipulation of second messengers in tubules

Clearly, it is desirable to be able to manipulate the major second messengers (cGMP, cAMP and Ca^{2+}) in specific cells in tubules, or indeed in any cell type. This project investigates the feasibility of performing such manipulation, by ectopic expression of receptors under spatial and temporal control.

1.4.1 Guanylate cyclases

Guanylate cyclases serve as receptors that produce cGMP in response to ligand binding. They fall into three distinct groups; those which are found on the plasma membrane, which recognise a variety of different peptides (rGCs); those in the cytoplasm, which recognise nitric oxide (sGCs); and vertebrate photoreceptor rod outer segment guanylate cyclases (ROS-GC) (Davies, 2000). The ROS-GCs differ from rGCs in that they are not regulated by extracellular ligands. Instead, they are regulated intracellularly by Ca^{2+} via Ca^{2+}-binding proteins (Goraczniak *et al*, 1998; Kumar *et al*, 1999).

The rGCs possess a single transmembrane domain, separating the protein in to an extracellular ligand binding domain and an intracellular region consisting of an intracellular kinase homolgy domain (KHD) and an intracellular cyclase catalytic domain (Chinkers *et al*, 1989). However, it is proposed that a homodimer is the minimum catalytic element of membrane guanylate cyclases. The cytoplasmic forms consist of two different subunits (α and β), each of which contains a cyclase catalytic domain (Garbers and Lowe, 1994). The known peptide ligands for the membrane receptors fall into two families: natriuretic peptides (atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP)); or heat-stable enterotoxins/guanylins. The membrane guanylate cyclases differ most greatly in the N-terminal ligand-binding domain, and are classified according to their ligand specificities.

1.4.2 cGMP signalling & GC-A as a tool to specifically manipulate cGMP

We chose to use a rat membrane-bound guanylate cyclase (GC-A) as a tool to specifically manipulate intracellular cGMP ([$cGMP_i$]) *in vivo*. GC-A is a membrane form of guanylate cyclase and upon binding the peptide atrial natriuretic peptide (ANP) elevates intracellular levels of cGMP directly. ANP was discovered by de Bold *et al* (de Bold *et al*, 1981; de Bold, 1982) as an endogenous natriuretic factor. It is found primarily in atria as a preprohormone that is cleaved to a prohormone of 126 amino acids (Gardner *et al*, 1991). The carboxy-terminal 28 amino acids represent the circulating form of ANP (Thibault *et al*, 1985; Glembotski *et al*, 1988). Binding of ANP to renal receptors was first reported by Napier *et al*, 1984 using radiolabelled ANP and principally induces natriuresis and diuresis in the kidney, as well as induction of smooth muscle relaxation and inhibition of aldosterone synthesis from the adrenal gland (Drewitt and Garbers, 1994).

Maximal activation of GC-A by ANP requires the presence of ATP (Foster and Garbers, 1998). It has been shown that the kinase homology domain (KHD) functions as a regulatory element although it does not have protein kinase activity (Chinkers and Garbers, 1989; Potter and Hunter, 1999). The KHD conforms to the protein kinase consensus sequence in 30 out of 33 residues and the Gly-X-Gly-X-X-Gly consensus sequence of protein kinases is Gly-X-Gly-X-X-X-Gly in the rat guanylate cyclase (Chinkers *et al*, 1989). When the KHD was removed by deletion mutagenesis, the resulting ANP receptor retained guanylate cyclase activity, but the activity was ANP-independent and its stimulation by ANP was markedly reduced (Chinkers and Garbers, 1989). This result suggests that binding of ANP to the receptor evokes a conformational change in the KHD which allows derepression of guanylate cyclase activity. It has subsequently been suggested that dephosphorylation of the receptor mediates desensitisation to ANP. Abolishing the six phosphorylation sites by

conversion to alanine residues in the KHD results in loss of ability to be activated by ANP. This suggests that the receptor is active in its phosphorylated state, and dephosphorylation is the regulatory means by which receptor activity declines over time (Potter and Hunter, 1999; Potter and Garbers, 1992) (Figure 1.8).

It has been demonstrated in GC-A knock-out mice, that disruption of GC-A leads to mice with elevated blood pressure, which is not altered by either minimal or high salt diet (Lopez *et al*, 1995). This was not due to reduced ANP levels in plasma in response to acute volume expansion, which were similar in both wild type and GC-A deficient mice (Kishimoto *et al*, 1996). However, elevated urine, sodium and cGMP output by wild type mice in response to volume expansion was not seen in GC-A deficient mice, implying that GC-A is essentitial for ANP-induced acute regulation of natriuresis and diuresis.

It is critical to our approach that the tissue of interest (in this case Malpighian tubules) does not respond to the hormone(s) that activate the chosen receptor. Tubules have been shown not to respond to ANP (Dow *et al*, 1994a) and in addition there are no ANP-like peptides in the *Drosophila* genome, determined by BLAST search. This suggests that ectopic hs::GC-A or UAS::GC-A will have general utility, not just in tubules.

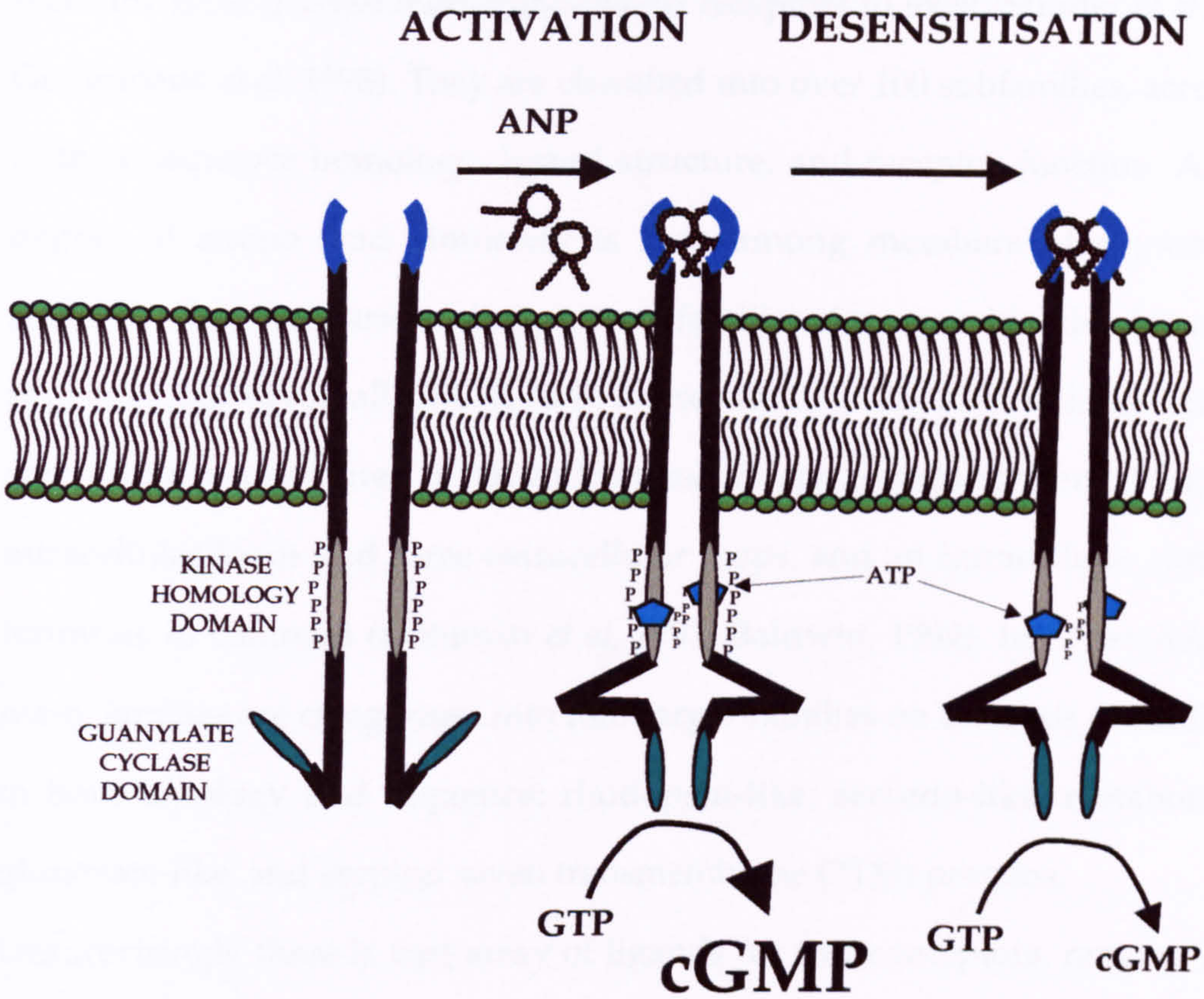

Figure 1.8: ANP-induced stimulation of GC-A catalyses formation of cGMP

GC-A is thought to exist as a dimer, or possibly higher-order configurations. An extracellular domain binds ANP, resulting in a conformational change that brings the guanylate cyclase domains into close contact with each other. This association catalyses formation of cGMP from GTP. At least four serine and two threonine residues in the kinase homology domain are phosphorylated in the absence of ligand, and dephosphorylation correlates with receptor desensitisation.

[Adapted from Silberbach and Roberts, Jr., 2001].

1.4.3 G protein-coupled receptors

G protein-coupled receptors (GPCRs) form a large superfamily with over 1000 different GPCRs estimated to exist in mammals (>1% of the genome!), making them the most diverse membrane-bound receptors to exist (Strader *et al*, 1994; Gudermann *et al*, 1995). They are classified into over 100 subfamilies, according to their sequence homology, ligand structure, and receptor function. A large degree of amino acid similarity is seen among members of a particular subfamily, but comparisons between subfamilies show considerably less, or no similarity. However, all GPCRs have an extracellular amino terminus, a central core domain constituted of seven transmembrane helices connected by three intracellular loops and three extracellular loops, and an intracellular carboxyl terminus in common (Dohlman *et al*, 1991; Baldwin, 1994). In *Drosophila*, the many families are categorised into four larger families on the basis of similarity in both topology and sequence: rhodopsin-like; secretin-like; metabotropic glutamate-like; and atypical seven transmembrane (7TM) proteins.

Unsurprisingly there is vast array of ligands for these receptors, ranging from hormones, neurotransmitters and growth factors, to odorants and light, and mutations in GPCRs have been observed that relate to a similarly wide range of disorders and diseases, from infertility to cancer. They are also estimated to be the target of more than 30% of the drugs used in clinical medicine. Thus, an understanding of GPCR function is an important goal.

When a GPCR is activated by an extracellular ligand, the receptor relays this information by activating the G protein with which it is associated. These G proteins are heterotrimers composed of, α, β, and γ subunits, and are classified based upon amino acid similarity of their α subunits. More than 20 distinct α subunits are known, which are subdivided into four families: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12}$ (Simon *et al*, 1991). Five β and eleven γ subunits have been described (Watson *et al*, 1994; Morishita *et al*, 1995, Ray *et al*, 1995), however β and γ

subunits are tightly associated and are usually regarded as one functional unit. In the inactive state, Gα subunits have GDP bound in a high-affinity binding pocket for guanine nucleotides, and are associated with the βγ subunit. Activated GPCRs catalyse the exchange of GDP for GTP, and the α subunit becomes dissociated from the βγ subunit. The Gα subunit had originally been regarded as the activator of signalling pathways, whereas the βγ subunit played a role in anchoring the Gα subunit to the membrane near the receptor. However, both GTP-Gα and βγ are signalling molecules, and modulate the activity of specific effector systems (Birnbaumer, 1992; Clapham and Neer, 1993; Neer, 1995). The Gα subunit controls the stimulation or inhibition of cAMP, or the release of Ca^{2+} from internal stores. The intrinsic GTPase activity of the Gα subunit hydrolyses GTP to GDP, thus rendering it inactive and the Gα subunit then reassembles with the βγ subunits. In order for this cycling between active and inactive states of the G protein, there needs to be receptor desensitisation to persistent stimulation. This can occur in numerous ways, from transcriptional and translational mechanisms, to the more rapid control of protein levels. The control of receptor stimulation at the protein level can involve regulation of the rate at which the receptor is internalised and degraded, or by covalent modifications and/or association with other regulatory proteins which block receptor-G protein association. An established mechanism for GPCR desensitisation is by feedback via kinase(s) activated by GPCR stimulation. Both cAMP-dependent kinase (cAK, stimulated by cAMP production), and Ca^{2+}-dependent kinase (PKC, stimulated by $G\alpha_q$ activation of phospholipase C) regulate GPCRs this way (Hausdorff *et al*, 1990; Sibley *et al*, 1987). Phosphorylation causes a conformational change in the receptor, which inhibits association with the G protein. This is a non-specific, or heterologous mechanism, because elevated cAK or PKC activity, caused by any agonist, will potentially phosphorylate any GPCR that has the appropriate phosphorylation

site. The major agonist-specific, or homologous, mechanism for rapid desensitisation of GPCRs involves a two step process. First, the activated receptor is phosphorylated by a G protein-coupled receptor kinase (GRK) and then the receptor binds β-arrestin protein, which blocks signalling to the G protein. This is reported to be an important process in the internalisation of GPCRs (for a review see Ferguson *et al*, 1996). β-arrestin binds with high-affinity to clathrin, suggesting that it acts as an adaptor in clathrin-coated vesicle-mediated endocytosis of receptor. Rapid desensitisation by internalisation of receptors is also rapidly reversible, with dephosphorylation of the receptor occuring in endosomes, before being returned to the plasma membrane (Figure 1.9).

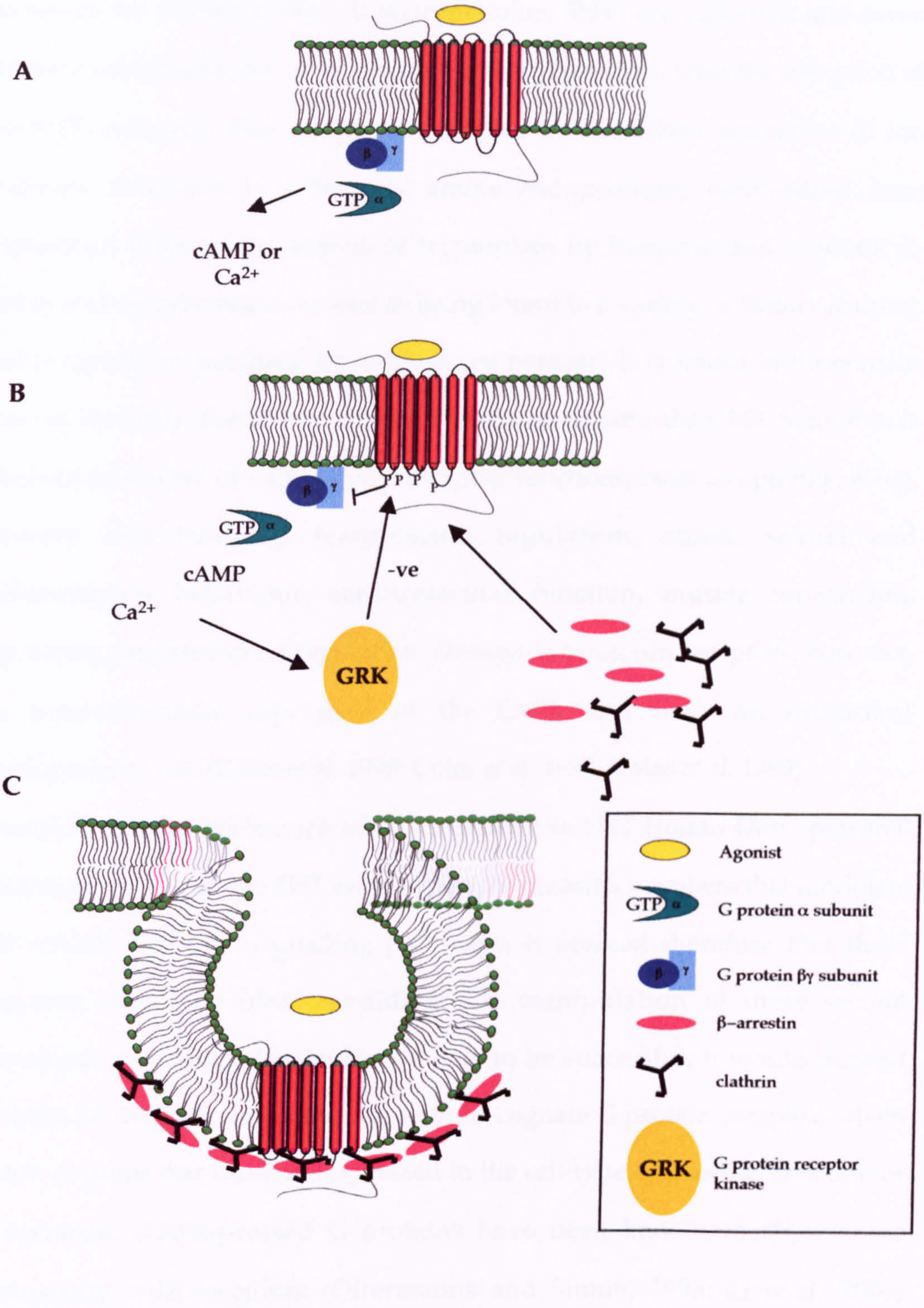

Figure 1.9: A model for G protein-coupled receptor desensitisation

A: The activated receptor stimulates production of cAMP or release of Ca^{2+}.

B: An activated G protein receptor kinase phosphorylates the activated receptor, which blocks further stimulation of the G protein. This in turn allows the association of β-arrestin and clathrin.

C: Clathrin assembly results in endocytosis of the receptor, where it can be dephosphorylated and recycled or degraded.

1.4.4 Serotonin (5HT) receptors

Receptors for serotonin (5-hydroxytryptamine, 5HT) are classified into seven different subfamilies, all of which are G protein-coupled, with the exception of the $5HT_3$ receptor; this receptor is coupled to the direct activation of ion channels. Serotonin is a biogenic amine endogenously synthesised from tryptophan through the actions of tryptophan hydroxylase and aromatic L-amino acid decarboxylase, as well as being found in a variety of dietary sources, and is rapidly metabolised by monoamine oxidase. It is found in three main areas of the body; the intestinal wall, blood vessels, and the CNS. Serotonin is involved in control of various physiological functions, such as appetite, sleep, memory and learning, temperature regulation, mood, sexual and hallucinogenic behaviour, cardiovascular function, muscle contraction, depression, and endocrine regulation. *Drosophila* serotonin receptors, however, are predominantly expressed in the CNS, and have an important developmental role (Colas *et al*, 1995; Colas *et al*, 1997; Colas *et al*, 1999).

Drosophila tubules are known to be insensitive to 5HT (Julian Dow, personal communication), and the 5HT receptor family contains members that modulate both cAMP and Ca^{2+} signalling pathways. It seemed therefore that these receptors would be ideal candidates for manipulation of these second messengers in tubules. However, if this is to be successful, it would depend critically on the receptor interacting with its cognate G protein complex, which in turn requires that these are expressed in the cell-type of interest. This cannot be assumed: overexpressed G proteins have been known to show some promiscuity with receptors (Offermanns and Simon, 1995; Li *et al*, 2000; Kostenis 2001). Accordingly, this study will go into some detail to demonstrate that the receptors used really do act exclusively on the second messenger of interest, and exclusively on the intended cell types. These are essential

validations of the method, and as will be seen, the transgenic receptors indeed behave as desired.

1.4.5 cAMP signalling & $5HT_{7Dro}$ as a modulator of cAMP

The manipulation of cAMP was achieved using a *Drosophila* receptor for serotonin, $5HT_{7Dro}$ (Witz *et al*, 1990; Saudou *et al*, 1992; see Table 1.1 and review by Tierney (2001) for nomenclature). This receptor is a G protein-coupled receptor, which upon activation, stimulates adenylate cyclase to catalyse the formation of cAMP when expressed in insect cell lines (Obosi *et al*, 1996) (Figure 1.10 and Table 1.2). It has been implicated in regulation of circadian rhythms, based on the presence of a Gly-Ser repeat in the N-terminal tail of the receptor. This motif is an attachment site for glycosaminoglycans found in biological clock genes, such as *period*.

Serotonin does not physiologically affect *Drosophila* tubules, however adenylate cyclase activity can be raised by the activator forskolin, confirming the presence of adenylate cyclase(s) in this tissue, and suggests that the $5HT_{7Dro}$ is a good candidate as a receptor to specifically modulate cAMP.

The role of cAMP in cells can be diverse, but the simplistic model is that cAMP is generated and acts to raise cAK activity, which is its main effector. The actions of cAK then depend on the particular cell type and appropriate substrates containing the cAK consensus phosphorylation site. Phosphodiesterase activity increases in response to elevated cAMP levels, and hydrolyses cAMP to 5′-AMP. This is rather more complicated than a simple linear pathway that is dampened by degradation of cAMP, and this is largely due to differential expression and compartmentalisation of the adenylate cyclase and cAMP-dependent phosphodiesterase subtypes in a particular cell.

Adenylate cyclases belong to a large family encoded by at least nine independent genes, with the different subtypes only expressed in distinct

Original name	Revised name	Accession *n*	Reference
5HT-dro1	$5HT_{7Dro}$	M55533	Witz *et al*, 1990
5HT-dro2A	$5HT_{1ADro}$	Z11489	Saudou *et al*, 1992
5HT-dro2B	$5HT_{1BDro}$	Z11490	Saudou *et al*, 1992
$5HT_{2Dro}$	$5HT_{2Dro}$	X81835	Colas *et al*, 1995

Table 1.1: *Drosophila* 5HT receptor nomenclature

[Adapted from Tierney, 2001].

Receptor	Transduction	Agonists	Antagonists	Reference
$5HT_{7Dro}$	Increase cAMP	5HT (4) 8-OH-DPAT (8)	dihydroergocryptine (1) d-butaclamol (2) methysergide (3) prazosin (5) l-butaclamol (6) yohimbine (7)	Saudou *et al*, 1992
$5HT_{1ADro}$	Decrease cAMP Increase $InsP_3$	5HT (6) 8-OH-DPAT (8)	dihydroergocryptine (1) prazosin(2) d-butaclamol (3) methysergide (4) l-butaclamol (5) yohimbine (7)	Saudou *et al*, 1992
$5HT_{1BDro}$	Decrease cAMP Increase $InsP_3$	5HT (5) 8-OH-DPAT (7)	dihydroergocryptine (1) d-butaclamol (2) prazosin(3) methysergide (4) yohimbine (5) l-butaclamol (8)	Saudou *et al*, 1992
$5HT_{2Dro}$	?	5HT (4) N-acetyl-5HT (6) α-methyl-5HT (8) 2-methyl-5HT (13) tryptamine (13) 1-methyl-5HT (15) quipazine (17) 5-MeOT (17)	ritanserin (1) ketanserin (2) pizotifen (3) setoperone (5) spiperone (6) cyproheptadine (6) TFMPP (6) mesulergine (6) methysergide (7) methiothepine (7) rauwolsine (9) buspiron (10) yohimbine (11) bufotenine (12) mianserin (14) cis-flupenthixol (16)	Colas *et al*, 1995

Table 1.2: Pharmacological profile of heterologously expressed *Drosophila* 5HT receptors

Table includes all serotonergic ligands tested in rank order of potency (except for $5HT_{2Dro}$ where the 25 most potent compounds are listed). Numbers in parentheses after each compound indicate the order of potency of agonists relative to antagonists; equally potent compounds are assigned the same number. Abbreviations: 8-OH-DPAT, (±)-8-hydroxy-2-(di-*n*-dipropylamino) tetralin; 5-MeOT, 5-methoxytryptamine; TFMPP, N-(-3-trifluoromethylphenyl)piperazine.

[Adapted from Tierney, 2001].

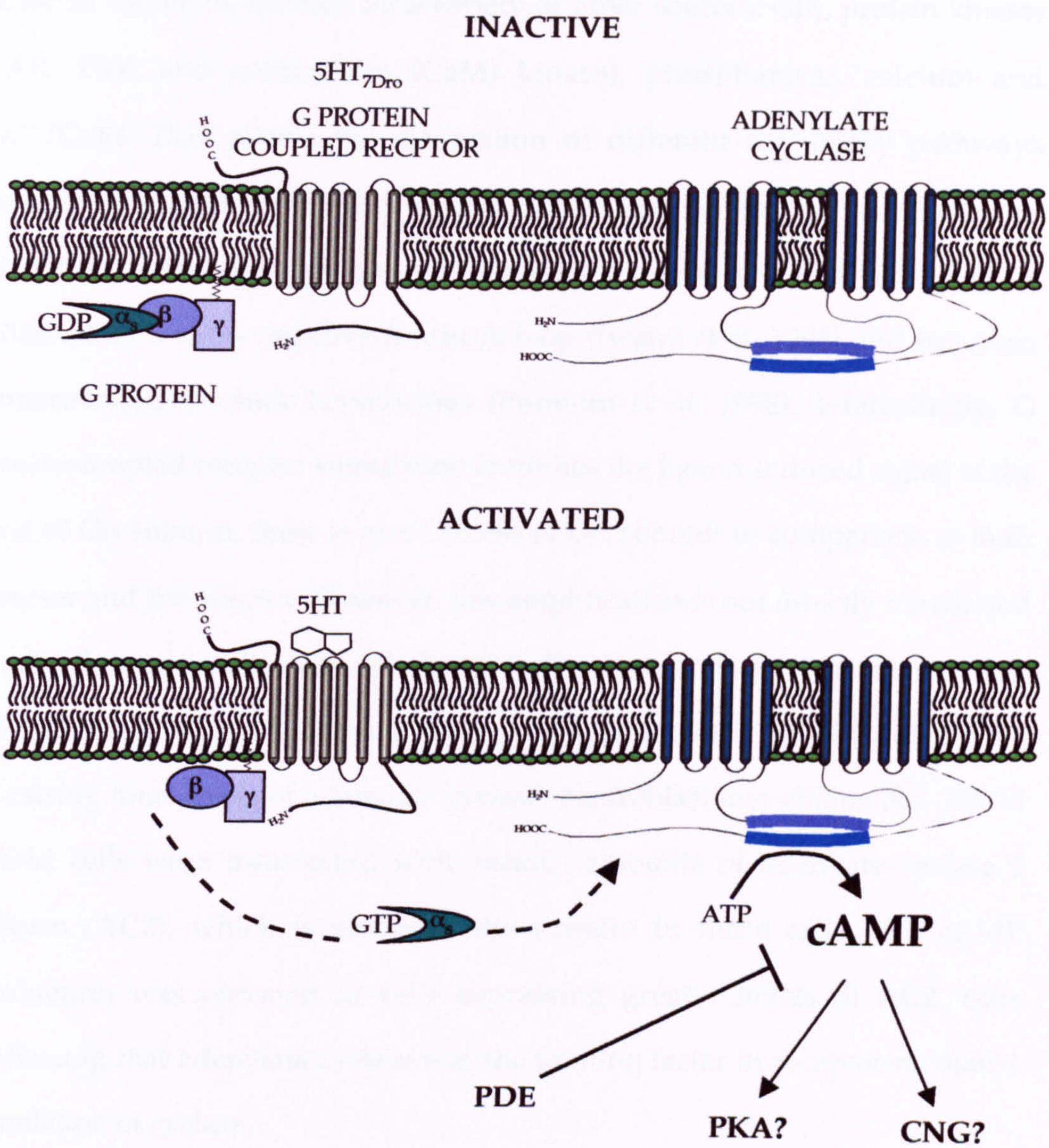

Figure 1.10: Activation of the $5HT_{7Dro}$ G protein-coupled receptor stimulates cAMP production

Binding of 5HT to the $5HT_{7Dro}$ receptor stimulates the heterotrimeric G protein with which it is associated to exchange GDP for GTP. This exchange allows the α_s subunit to dissociate from the $\beta\gamma$ subunits, and consequently stimulate adenylate cyclase to catalyse the formation of cAMP from ATP. The intrinsic GTPase activity of the α_s subunit hydrolyses GTP to GDP, thus allowing it to reassociate with the $\beta\gamma$ subunits in its inactive state. In the absence of 5HT, almost all the $G\alpha_s$ is in the inactive GDP-bound form.

tissues. In addition to activation by $G\alpha_s$ and inhibition by $G\alpha_i$, the different isoforms can be modulated by a variety of other sources: $G\beta\gamma$, protein kinases (cAK, PKC and calmodulin (CaM) kinase), phosphatases, calcium and Ca^{2+}/CaM. This allows the integration of different regulatory pathways through cross-talk with other signal transduction pathways. Inhibition of adenylate cyclase by cAK presents a model whereby desensitisation can be achieved by a short negative-feedback loop (Iwami *et al*, 1995) and has been demonstrated in chick hepatocytes (Premont *et al*, 1992). Interestingly, G protein-coupled receptor stimulation amplifies the ligand-induced signal at the level of $G\alpha$ subunit: there is great excess of $G\alpha$ subunit in comparison to both receptor and the effector. However, this amplification is not directly transferred to adenylate cyclase activity. Work by MacEwan *et al* (1996) demonstrated that the maximal hormonal stimulation of cAMP production could be potentiated by raising total levels of adenylate cyclase. Neuroblastoma-glioma NG 108-15 hybrid cells were transfected with various amounts of adenyate cyclase 2 isoform (AC2), which is not normally present in these cells, and cAMP production was elevated in cells expressing greater levels of AC2, thus confirming that adenylate cyclase was the limiting factor in receptor-mediated stimulation of cyclase.

The profile of the cAMP signal can also be due to the phosphodiesterases present in a tissue/cell. Phosphodiesterases are a family of enzyme that has expanded rapidly in recent years: at present there are at least eleven subfamilies of mammalian phosphodiesterase, most of which contain more than one gene product. They are grouped according to their regulator and substrate specificities and genetically on the basis of sequence homology (Essayan, 2001). Regulation of PDEs can occur by Ca^{2+}/CaM, cGMP, cAMP, cGK, cAK , insulin, leptin and light. The function of phosphodiesterases also varies between families: this is determined by both tissue distribution and by which cyclic

nucleotide the phosphodiesterase hydrolyses (PDEs 4, 7 and 8 hydrolyse cAMP; PDEs 5, 6 and 9 hydrolyse cGMP; and PDEs 1, 2, 3, 10 and 11 will hydrolyse both cAMP and cGMP). The diversity in tissue distribution and regulators of PDEs creates great potential for cross-talk between pathways that elevate cGMP production and pathways that function *via* cAMP. Indeed, there appears to be a degree of cross-talk already identified in tubules: the addition of cAMP to tubules increases V-ATPase activity, as does that of cGMP, indicating that regardless of prior events leading to elevated cyclic nucleotide, they have at least one downstream effector in common.

1.4.6 Ca^{2+} signalling & $5HT_{1A\,Dro}$ as a modulator of $[Ca^{2+}]_i$

The manipulation of intracellular Ca^{2+} was achieved using a *Drosophila* receptor for serotonin, $5HT_{1ADro}$ (Saudou *et al*, 1992; see Table 1.1 and review by Tierney (2001) for nomenclature). As with the $5HT_{7Dro}$ receptor, this is a G protein coupled receptor. However, it does not couple to adenylate cyclase activation, but instead stimulates production of inositol 1,4,5- trisphosphate ($InsP_3$) *via* coupling to $G\alpha_q$ when expressed in mammalian cell lines (Saudou *et al*, 1992; Table 1.2). $InsP_3$ is generated by the hydrolysis of the lipid precursor phosphatidylinositol 4,5-bisphosphate ($PtdIns(4,5)P_2$) to diacyl glycerol (DAG) and $InsP_3$ by phospholipase C (PLC) upon its activation by $G\alpha_q$ (Figure 1.11). $InsP_3$ diffuses through the cytosol and binds to $InsP_3$ receptors ($InsP_3R$) found on the membrane of internal stores (such as the endoplasmic reticulum). These receptors are found as six transmembrane-spanning tetramers that form Ca^{2+} channels, allowing Ca^{2+} to be released from stores into the cytosol, although are also sometimes found on the plasma membrane. They have also been implicated as mediators of Ca^{2+} release from secretory vesicles and the Golgi apparatus (Petersen, 1996; Pinton *et al*, 1998). Three distinct genes (I-III) have so far been identified that encode mammalian $InsP_3$ receptors, with two additional

isoforms (IV and V) proposed to exist, based on partial sequence information. However, alternative splicing generates further heterogeneity amongst these isofoms. There does not appear to be such diversity in *Drosophila*, with only one $InsP_3$ receptor gene identified (Yoshikawa *et al*, 1992). Nevertheless, there is a family of evolutionarily related receptors, named ryanodine receptors (RyR), found in both mammals and *Drosophila* that are also involved in release of Ca^{2+} into the cytosol, which adds further heterogeneity to the mechanisms that can influence Ca^{2+} release.

Cytosolic Ca^{2+} is kept low (~0.1μM) under resting conditions, but under hormonal stimulation it may rise to ~1μM (Berridge, 1993). This is achieved by Ca^{2+} release from internal stores, which can also mediate Ca^{2+} entry from extracellular fluid (known as capacitative calcium entry, through store operated channels (SOCs)); both have far higher concentrations of Ca^{2+} than the cytosol (Berridge, 1995). $InsP_3R$ and RyR both display calcium-induced calcium release (CICR), whereby Ca^{2+} released from one receptor can excite its neighbours, thus triggering a wave of Ca^{2+} release throughout the cytoplasm (Berridge 1997; Berridge *et al*, 2000). At rest, $InsP_3$ is actually also capable of sensitizing $InsP_3R$ to produce Ca^{2+} oscillations, known as sparks or puffs (Bootman *et al*, 2002).

Because of the high surrounding levels of Ca^{2+}, the cell has to continually pump Ca^{2+} out of the cytoplasm, using Ca^{2+}-ATPases on the plasma membrane and endoplasmic reticulum, maintaining two sources of Ca^{2+} to utilise as a signal. Cytosolic Ca^{2+} is relatively non-diffusable, due to the many high-affinity binding capabilities of relatively immobile proteins (Kasai and Petersen, 1994), and so a variety of different messages can be achieved from this second messenger, simply by the spatial and temporal control of Ca^{2+} release.

Ca^{2+} signalling and the Ca^{2+} channels present in *Drosophila* tubules have been examined in elegant detail previously (O'Donnell *et al*, 1996; Rosay *et al*, 1997; Terhzaz *et al*, 1999; MacPherson *et al*, 2000; MacPherson *et al*, 2001; Pollock *et al*,

submitted 2002). As described earlier, CAP_{2b} stimulation of tubules results in a principal cell-specific rise in $[Ca^{2+}]_i$ which in turn activates a Ca^{2+}/CaM-sensitive nitric oxide synthase (NOS) (Davies *et al*, 1997), whereas tubule stimulation with *Drosophila* leukokinin induces a rise in $[Ca^{2+}]_i$ only in stellate cells. Elevated fluid transport and $[Ca^{2+}]_i$ are both dependent on channels that allow Ca^{2+} entry into tubules; these are both abolished in calcium-free medium. Furthermore, in the absence of external calcium, tubules treated with the Ca^{2+}-ATPase inhibitor thapsigargin only display elevated $[Ca^{2+}]_i$ in stellate cells, which suggests that principal cells do not have a thapsigargin-sensitive intracellular calcium pool, or that it is too small or labile to be detected (Rosay *et al*, 1997). This implies that the $[Ca^{2+}]_i$ rise seen in response to CAP_{2b} is by Ca^{2+} entry through basolateral membrane Ca^{2+} channels. It also suggests that different cell subtypes have different Ca^{2+}-cycling mechanisms. Expression of the $5HT_{1ADro}$ receptor as a specific modulator of $[Ca^{2+}]_i$ adds a new dimension to studying Ca^{2+} cell specific signalling events, with serotonin creating no background activity in tubules. Thus, this approach removes the complication of cross-activation that is evident in pre-existing fluid transport mechanisms.

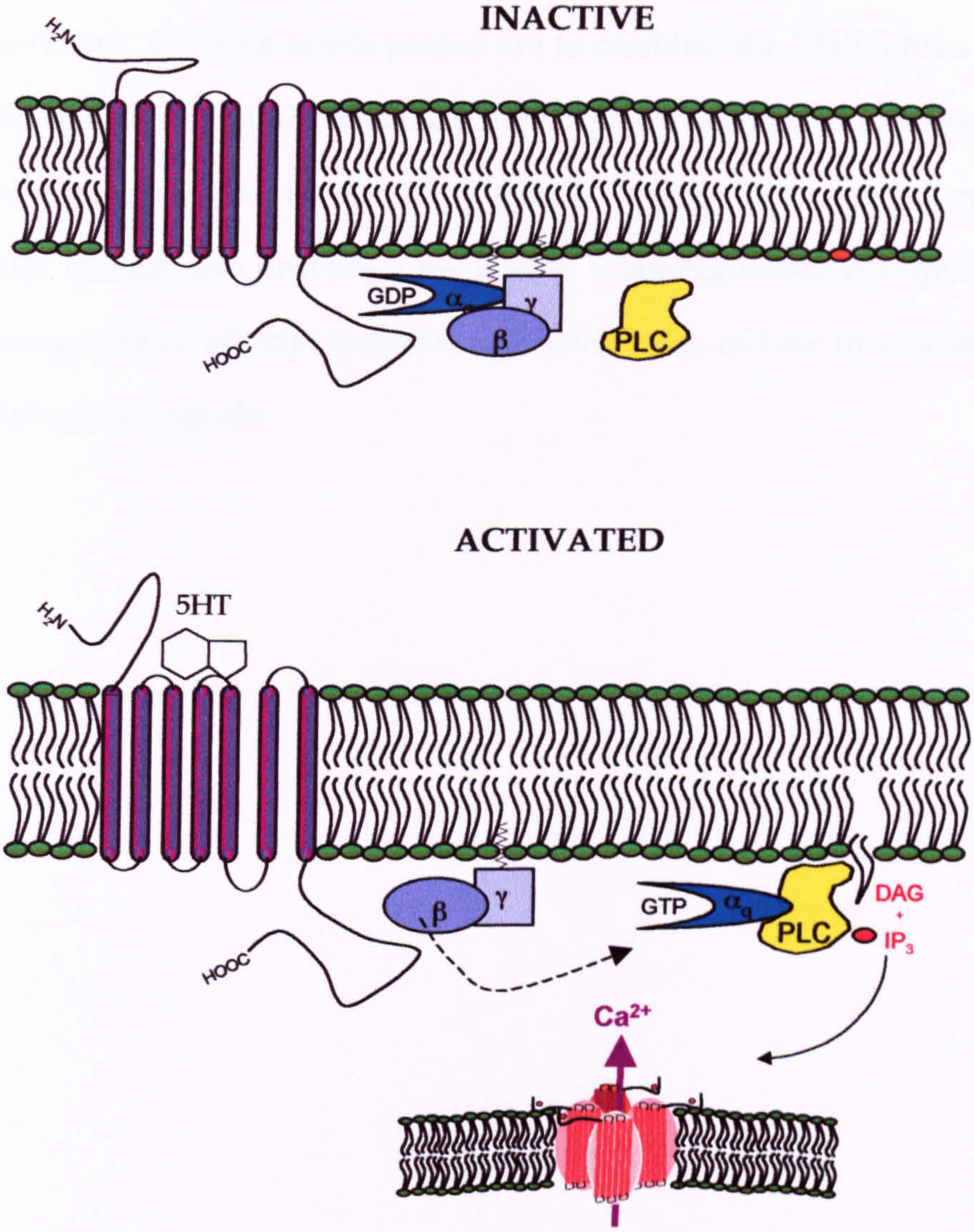

Figure 1.11: Activation of the $5HT_{1ADro}$ G protein-coupled receptor stimulates Ca^{2+} release from the ER

Binding of 5HT to the $5HT_{1ADro}$ receptor stimulates the heterotrimeric G protein with which it is associated to exchange GDP for GTP. This exchange allows the Gα subunit to dissociate from the βγ subunits, and consequently activate phospholipase C (PLC). Phospholipase C then hydrolyses the lipid precursor phosphatidylinositol 4,5-bisphosphate (PIP_2) to give both diacyl glycerol (DAG) and inositol (1,4,5)-trisphosphate ($InsP_3$ or IP_3). The latter then binds to the $InsP_3$ receptor (IP_3R) to mobilise Ca^{2+} from internal stores (ER).

The intrinsic GTPase activity of the Gα subunit hydrolyses GTP to GDP, thus allowing it to reassociate with the βγ subunits in its inactive state. In the absence of 5HT, almost all the $G\alpha_s$ is in the inactive GDP-bound form.

1.5 Specific aims

In summary, the aims of this project are to establish the Malpighian tubule as a model epithelium within which the *in vivo* pharmacology of receptors and their downstream effectors can be investigated. Ectopic expression of receptors in tubules should also provide a method to unambiguously manipulate second messengers in an attempt to further our knowledge of how this tissue processes and integrates signals.

Chapter 2

Materials

&

Methods

2.1 *Drosophila melanogaster*

2.1.1 *Drosophila* stocks

Listed below are the *Drosophila* lines utilised in this study and their application.

Strain	Purpose/Use	Source/ Citation
Oregon R	Control for fluid secretion assays. Control for Western analysis. cDNA production.	Standard laboratory stock.
w^{1118}	Control for fluid secretion assays & cyclic nucleotide assays. Background strain for microinjection.	Hazelrigg *et al*, 1984.
c42	GAL4 enhancer trap line. Expression of GAL4 in principal cells of main segment in tubules.	Sözen, 1996
c724	GAL4 enhancer trap line. Expression of GAL4 in stellate cells of main segment in tubules.	Sözen *et al*, 1997
c710	GAL4 enhancer trap line. Expression of GAL4 in stellate cells of main segment in tubules.	Sözen *et al*, 1997
hs::GC-A	P-element insertion of a rat guanylate cyclase gene (GC-A) under control of Hsp 70 heat shock promoter. Used to modulate cGMP levels in tubules.	Generated for this study
UAS::GC-A	P-element insertion of a rat guanylate cyclase gene under control of GAL4 responsive *U*pstream *A*ctivating *S*equence. Used to modulate cGMP levels in tubules.	Generated for this study
hs::$5HT_{7Dro}$	P-element insertion of $5HT_{7Dro}$ receptor under control of Hsp 70 heat shock promoter. Used to modulate cAMP levels in tubules.	Generated for this study
UAS::$5HT_{7Dro}$	P-element insertion of $5HT_{7Dro}$ receptor under control of GAL4 responsive *U*pstream *A*ctivating *S*equence. Used to modulate cAMP levels in tubules.	Generated for this study
UAS::$5HT_{1ADro}$	P-element insertion of $5HT_{1ADro}$ receptor under control of GAL4 responsive *U*pstream *A*ctivating *S*equence. Used to modulate $[Ca^{2+}]_i$ in tubules.	Kind gift of Frédéric Saudou

c42-aeq	P-element insertions of aequorin transgene, under control of UAS, and GAL4 enhancer trap (c42). Directs expression of aequorin to principal cells of tubules, for cell-specific calcium measurements.	Rosay *et al*, 1997
c710-aeq	P-element insertions of aequorin transgene, under control of UAS, and GAL4 enhancer trap (c710). Directs expression of aequorin to stellate cells of tubules, for cell-specific calcium measurements.	Rosay *et al*, 1997
hsGAL4-aeq	P-element insertions of aequorin transgene, under control of UAS, and GAL4, under control of Hsp70 heat shock promoter. Allows global expression of aequorin for calcium measurements.	Rosay *et al*, 1997

Table 2.1: *Drosophila* strains used in this study

2.1.2 *Drosophila* rearing

Flies were maintained in standard conditions (Ashburner, 1989) on a 12 h dark : 12 h light cycle in vials of standard cornmeal/yeast/agar *Drosophila* medium at 25°C. If large quantities of flies were required, rearing was in large bottles on standard medium.

2.1.3 *Drosophila* crossing schemes

All hs::transgene, UAS::transgene and GAL4 lines were homozygosed in a w^{1118} background. Lines containing hs::transgene were used 12-18 h after a 45 min-1h heat-shock at 37°C, with no need for further crossing. For expression of a transgene under UAS control, UAS::transgene virgin females were collected and crossed with males from the appropriate GAL4 line, or *vice versa*. Under these conditions, all progeny contain one copy of each transgene.

2.1.4 *Drosophila* dissection

All dissection of tissues were performed on flies anaesthetised on ice in a small Petri dish, under a layer of Schneider's revised *Drosophila* medium (Gibco BRL) with two pairs of watchmaker's forceps (Biologie, #55).

2.2 Fluid Secretion Assays

This assay was carried out as previously described (Dow *et al*, 1994b). Adult *Drosophila* tubules were dissected out into Schneider's revised *Drosophila* medium (Gibco BRL) and pairs of tubules were transferred to a 9 µl drop of a 1:1 (Schneider's:*Drosophila* saline) mix (*Drosophila* saline, 117.5 mM NaCl, 20 mM KCl, 2 mM $CaCl_2$, 8.5 mM $MgCl_2$, 10.2 mM $NaCO_3$, 4.3 mM Na_2HPO_4, 8.6 mM HEPES, pH=6.9, with 20 mM glucose added before use).

One tubule was drawn from the drop and looped around a pin, ensuring that the ureter (from where secretion issues) was out of the drop.

Every ten min the droplets excreted from the ureter were removed with a fine rod and their diameter measured using an eyepiece graticule.

When agonists were to be added, they were first diluted in Schneiders:*Drosophila* saline, if required, before addition of 1 µl of the compound to the bubble. Results were analysed using Excel 5.0 (Microsoft), where secretion rate was calculated in nl/min from the volume of fluid secreted in 10 min.

2.3 Nucleic Acid Isolation

2.3.1 Plasmid DNA isolation

Small scale plasmid DNA preparation was performed with the Qiaprep Spin Miniprep Kit (Qiagen) following manufacturer's instructions.

Large scale plasmid DNA preparation was performed with the Qiaprep Spin Endotoxin free Maxiprep kit (Qiagen) following manufacturer's instructions.

2.3.2 Preparation of genomic DNA

Single fly genomic DNA preparations for utilisation in standard PCR reactions were performed according to Gloor and Engels, 1991. Briefly, an anaesthetised single fly was placed in a 0.5 ml Eppendorf tube. 50 µl of homogenisation buffer (10 mM Tris-HCl (pH 8.3), 1 mM EDTA, 25 mM NaCl, 200 µg/ml proteinase K) was drawn up into the pipette tip and the fly squashed by the tip, without expelling the liquid.

Once the fly was suitably squashed, the liquid was expelled and the reaction incubated for 30 min at 37°C. The proteinase K was inactivated by heating the reaction for 1.5 min at 95°C. 1 µl of the reaction was then utilised for PCR. DNA from squashed flies could be maintained for several months at -20°C.

Genomic DNA from 30 flies was prepared for inverse PCR reactions (section 2.10.4). Flies were frozen in liquid nitrogen, then ground in 200 µl homogenisation buffer (100 mM Tris-HCl (pH 7.5), 100 mM EDTA, 100 mM NaCl, 0.5% (w/v) SDS). A further 200 µl homogenisation buffer was added and the ground tissue suspension was incubated for 30 min at 65°C. 800 µl LiCl/KAc solution (1 part 5 M KAc : 2.5 parts 6 M LiCl) was added and the homogenate was transferred to ice for 10 min. After this incubation, the homogenate was centrifuged at 13 000 rpm for 15 min and 1 ml of the supernatant was transferred to a new Eppendorf tube, with care taken to avoid transfer of solid tissue. Nucleic acid was precipitated by adding 600 µl

isopropanol to the supernatant, which was then vortex mixed and centrifuged at 13 000 rpm for 15 min. Supernatant was aspirated, the tube given a pulse centrifugation, and remaining supernatant aspirated. The remaining pellet of genomic DNA was washed with 70% ethanol and allowed to dry thoroughly before resuspending in 150 µl TE buffer. DNA was stored at –20°C.

2.3.3 Ethanol precipitation of DNA

When it was necessary to concentrate or clean DNA, ethanol precipitation was carried out. 0.1 volume 3 M Na acetate, pH 5.3, was added to samples followed by 2.5 volumes of 95% ethanol. The precipitation was then allowed to occur on ice for at least 1 h before DNA was pelleted by centrifugaton for 30 min at 15 000 rpm. The pelleted DNA was washed in 70% ethanol twice and resuspended in an appropriate volume of distilled water or TE buffer (Sambrook *et al*, 1989).

2.3.4 Quantification of nucleic acids

Nucleic acid concentrations were estimated by spectrophotometry at 260 nm and 280 nm, where an OD of 1 at 260 nm corresponds to 50 µg/ml of double-stranded DNA and 40 µg/ml of single-stranded DNA and RNA. Readings were zeroed with the solution in which the samples had been diluted. The ratio of $A_{260/280}$ provided an estimation of nucleic acid purity. Values between 1.8 and 2.0 indicated pure preparations (Sambrook *et al*, 1989).

Where concentrations of DNA were extremely small, the quantity of DNA was estimated by dropping 1 µl onto a 1% agarose (w/v in H_2O) plate containing 0.5 µg/ml ethidium bromide (EtBr), allowing it to dry and comparing its intensity to that of a series of known DNA standards (6, 12, 25, 50, 100 and 200 ng) under UV illumination at 254 nm on a transilluminator.

2.4 Restriction Digests and Electrophoresis

2.4.1 Restriction digests

DNAs were restricted in single strength REact® buffer appropriate to the restriction enzyme being used (all Gibco BRL). Where digests involving two enzymes with non-compatible buffers were necessary, a single digest was performed for 1 h at 37°C in the buffer of lower salt strength and then the enzyme was heat inactivated at 65°C for 10 min. At this point, an appropriate amount of salt solution was added to adjust to the correct conditions for the second enzyme, if possible. Otherwise, the singly digested DNA was purified by ethanol precipitation and resuspended in the appropriate single strength buffer, before commencing with the second restriction digest for 1 h at 37°C. Typical amounts of DNA in a restriction digest were 1 μg of plasmid DNA and 5 μg of genomic DNA.

2.4.2 Agarose gel electrophoresis of DNA

DNAs were separated in 1% agarose in 1x TBE (90 mM Tris, 90 mM boric acid (pH 8.3), 2 mM EDTA) containing 0.1 μg/ml EtBr, as described in Sambrook *et al*, 1989, using 1x TBE as the electrophoresis buffer. Sizes were compared to a 1 kb ladder (Gibco BRL). Prior to loading 3 μl of loading dye (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% (v/v) glycerol in water) was added to the samples.

2.5 Gel extraction of DNA

DNAs were extracted from agarose gels using the QIAquick Gel Extraction kit (Qiagen) following the protocol provided.

2.6 Polyacrylamide gel electrophoresis for DNA sequencing

Denaturing polyacrylamide gel electrophoresis was used to separate the products of sequencing reactions (section 2.12.1). Sequencing gels were composed of 6% (w/v) acrylamide/bis (acrylamide:N, N'-methylenebisacrylamide, 19:1), 7 M urea in 1x TBE (provided as a complete solution by Anachem). Gels were polymerised via the addition of 1 ml 10% (w/v in H_2O) APS (ammonium persulphate) and 50 µl TEMED in a Bio-Rad sequencing apparatus. Gels were allowed to set for at least half an hour before use and pre-run to reach the desired temperature (50°C). The samples to be electrophoresed were denatured for 5 min at 90°C and snap-chilled on ice before loading. Gels were run for two to four hs (in 1x TBE) at 50°C depending on the size of DNA to be resolved before drying down onto Whatman 3MM paper under vacuum.

2.7 Ligation of DNA

Ligation of DNA was typically performed with a ratio of 3:1 insert to vector, using a maximum of 200 ng DNA in 1x ligation buffer, with 2 Weiss units of T4 DNA ligase (Gibco BRL). Reactions were left for several h at room temperature.

2.8 Transformation into *E. coli*

2.8.1 *E. coli* strains

Strain	Genotype
TOP10 competent cells (Invitrogen).	(F⁻ *mcr* A, Δ(*mrr-hsd* RMS-*mcr*BC), ϕ80*lacZ* ΔM15, Δ*lacX74*, *rec*A1, *deo* R, *ara*D139, Δ (*ara-leu*)7697, *gal*U, *gal*K, *rps*L, (Str^R), *end*A1, *nup* G).
DH5α™ subcloning efficiency competent cells (Gibco BRL).	(F⁻ ψ 8 0 d*lac* ZDM15, Δ(lacZYA-*arg*F), U169, *deo* R, *rec*A1, *end*A1, *hsd* R17 (r_K^-, m_K^+), *pho* A, *sup* E44, λ-, *thi*-1, *gyr*A96, *rel* A1).

Table 2.2: *E. coli* strains used in this study.

Chemical transformation of competent cells was performed by adding 10-100 ng plasmid DNA to a 50 µl aliquot of cells on ice and stirring gently with a pipette tip. The cells were kept on ice for a further 30 min, then heat shocked by transferring to a 42°C water bath for 30 s. Cells were returned to ice for 2 min before the addition of 250 µl L-Broth medium and incubation on a flat bed shaker for 1 h at 37°C. A 200 µl aliquot of cells was then spread on agarose plates containing antibiotic (typically 100 µg/ml ampicillin).

2.8.2 Detection of β-galactosidase using X-gal

If blue/white selection of colonies from plasmids containing the *lacZ* gene was required, plates were spread with 20 µl 40mg/ml X-gal and allowed to dry thoroughly, prior to spreading of cells.

2.8.3 Storage of bacterial cultures

0.5 ml of bacterial culture was added to 1 ml of a 2% peptone, 40% glycerol solution (in H_2O) before being frozen under liquid nitrogen. Frozen stocks were stored at –70°C.

2.9 Oligonucleotide synthesis

Oligonucleotides were synthesised by the Gibco BRL custom primer service and by MWG. These were resuspended in TE buffer to a concentration of 100 μM. All primers were stored at -20°C. A list of all primers used in this is provided in Appendix 1. Primer concentrations were determined by UV spectrophotometry if required.

2.10 Polymerase Chain Reaction

2.10.1 Standard PCR

Standard PCR protocols were used for amplification of DNAs. Amounts of template DNA varied, with 0.5 μg genomic template DNA used per reaction and 0.1 μg or less of plasmid template. For reactions using Boehringer Mannheim *Taq* polymerase, dNTPs (Boehringer Mannheim) were added at 0.125 mM each to single strength PCR buffer, left and right primers at a concentration of 0.5 μM with 1 u of *Taq* polymerase. When Applied Biosystems Reddy Load Mix *Taq* was used, only template and primers at the same concentrations as above were added to the pre-aliquoted mix.

Cycling was performed in thin walled PCR tubes in a Hybaid OmnE, Hybaid PCR Sprint machine, or Hybaid Gradient PCR machine.

Cycling procedures were typically:

94°C for 1-2 min to ensure denaturation of template.

20-30 cycles of :

Denature at 94°C, 30 sec

Anneal at 48-60°C, 30 sec

Extend at 72°C, 1 min

then a final 2 min extension step of 72°C.

Annealing temperatures depended on the primers used.

2.10.2 High Fidelity PCR

When error free cloning of an open reading frame was required, a proof-reading DNA polymerase was used for PCR. The polymerase of choice was either *Pfu* (Promega) or a combination of *Taq* and *Pwo* (Roche).

Cycling procedures were typically :

94°C for 4-5 min to ensure denaturation of template.

10 cycles of :

Denature at 94°C, 15 sec

Anneal at 55-68°C, 30 sec

Extend at 68°C or 72°C, 1 min per kilobase DNA

Followed by 15-20 cycles of :

Denature at 94°C, 15 sec

Anneal at 55-68°C, 30 sec

Extend at 68°C or 72°C, 1 min per kilobase DNA + an additional 5 sec per cycle

Then a final 10 min extension step at 72°C.

Annealing temperatures depended on the primers used, and extension temperature depended on the proof-reading enzyme used.

2.10.3 Reverse transcriptase PCR

Reverse transcriptase PCR was performed as previously described (Dow *et al*, 1994a). PolyA$^+$ RNA was obtained using the magnetic Dynabeads mRNA DIRECT kit (Dynal®) according to manufacturers instructions. Heads (5) or tubules (80), were used in the extraction. Tissues were ground in Treff tubes with matching homogeniser.

Once the mRNA was extracted a reverse transcriptase reaction was set up.

This reaction contained;

0.2 mM of each dNTP

40 u RNAsin (Promega)

10 mM dithiotheitol (DTT)

1x first strand buffer,

and H_2O in a volume of 18 µl.

2 µl Superscript™ II RNase H- Reverse Transcriptase (Gibco BRL) was then added to start the reaction.

Reactions were incubated at 42°C for >30 min, with occasional tapping to resuspend the beads. The beads were then collected using the Dynal MPC magnet, washed and suspended in 20 µl TE buffer, the suspension being stored at -20°C. 1 µl of the Dynabead solution was sufficient template for a standard PCR reaction.

2.10.4 Characterisation of P-element insertion sites by inverse PCR

Inverse PCR is a method for mapping a P-element insertion within the genome of *Drosophila* (Dalby *et al*, 1995).

Using outward facing primers corresponding to sequence at either the 5' or the 3' end of the P-element, adjacent genomic DNA can be amplified (see figure 2.1). Template is generated by digestion of the P-element and genomic DNA with a restriction enzyme that cuts genomic DNA roughly every kilobase, and cuts the P-element just downstream (5' end) or upstream (3' end) of the primer sites. The digested DNA was then ligated and used in a PCR reaction with appropriate primers.

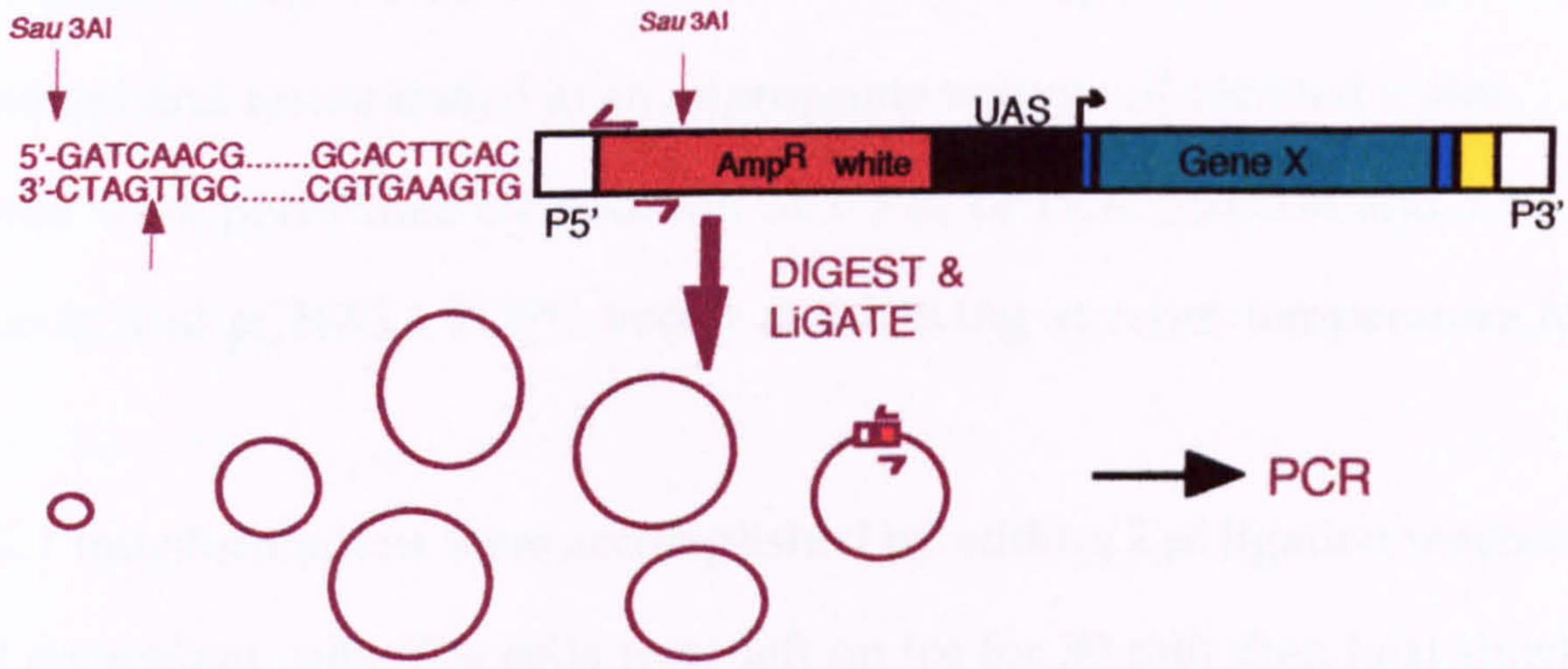

Figure 2.1 Schematic representation of inverse PCR

Genomic DNA was prepared by the 30 fly method described in section 2.3.2. 10 µl genomic DNA, roughly equivalent of two flies, was restriction digested with 10 units of *Sau*3AI or *Msp*I in the presence of 100 µg/ml RNase A. Digests were incubated for 2.5 h at 37°C. The restriction enzyme was then heat inactivated for 20 min at 65°C. Ligation of digested genomic DNA was performed as described in (section 2.7). The ligated DNA was then ethanol precipitated, the pellet was washed in 70% ethanol and dried, before

resuspension in 150 µl TE buffer (DNA concentration of approximately 1/150 th of a fly per µl). PCR of ligated genomic DNA was performed as in section 2.10.1.

2.11 Cloning of PCR products

PCR products were cloned using the Invitrogen TOPO TA cloning kit into pCR®2.1 TOPO vector according to manufacturers instructions and transformed into TOP10 competent cells. When PCR fragments generated by *Pfu* DNA polymerase were used, addition of overhanging adenine residues was necessary before being added to the reaction. This was achieved by incubating the PCR fragment in 1X PCR buffer (Roche) with dATP (0.025 mM) and 1 unit *Taq* polymerase for 10 min at 72°C. The PCR fragment was then ethanol precipitated and resuspended in an appropriate volume of distilled water.

Ligations were performed by addition of 1-3 µl of PCR reaction and 1 µl salt solution to 1 µl pCR®2.1 TOPO vector and leaving at room temperature for 5 min.

pCR®2.1 transformations were accomplished by adding 2 µl ligation reaction to TOP10 competent cells. The cells were left on ice for 30 min then heat shocked at 42°C for 30 s. The cells were put back on ice for 2 min before adding 250 µl SOC medium then transformations were shaken on their side for 30 min at 37°C.

100 µl of the transformation was spread onto L-agar plates containing 100 µg/ml ampicillin and incubated overnight at 37°C. These plates contained X-gal. White transformants were removed as single colonies and were inoculated overnight (with shaking) at 37°C in 5 ml L-broth containing 100 µg/ml ampicillin.

2.12 DNA Sequencing

2.12.1 Manual

Double-stranded DNA sequencing was carried out using the T7 Sequenase version 2.0 DNA sequencing kit (Amersham) or the T7 Sequenase quick denature plasmid sequencing kit (Amersham) using the methods described by the manufacturer. Both employ the dideoxy chain termination method of DNA sequencing using ^{35}S-α-dATP (ICN).

2.12.2 Automated

A single stranded PCR was performed with template and primers supplied at 1 µg and 3.2 pmol respectively, with a PCR mix containing fluorescently labelled dideoxynucleotides. Samples were run on an agarose gel with the nucleotides being detected on an ABI automated DNA sequencer. Automated sequencing procedures and materials were carried out by the Glasgow University Molecular Biology Support Unit. Analysis was carried out with the Applied Biosystems automated sequence analysis programme (Autoassembler 2.0).

2.13 Germline Transformation of *Drosophila*

2.13.1 Embryo collection and preparation

500 flies (w^{1118}) between 3 and 7 days old were set up in an egg collection cage the night before embryos were required. The following day, egg collection plates with a thick spot of yeast paste were placed on the cage and changed frequently until sufficient eggs were produced for injection (> 30) in a 20-25 min period. The eggs were transferred to a slide and dechorionated with fine forceps under a light microscope and lined up on the edge of a slide which had a thin layer of glue (Scotch tape dissolved in heptane) along the edge. The embryos were placed with the posterior pole facing outwards, dehydrated for 1 min and covered with 10S halocarbon oil (Sigma).

2.13.2 Glass micropipette preparation

Glass micropipettes were pulled on a Campden Instruments moving-coil microelectrode puller, model 753, from borosilicate glass capillary tubes with internal filament (Clark Genetic Instruments) of dimensions 1.0 mm (O.D.) x 0.78 mm (I.D.), with the puller settings on,

pre-pull heating time	30
initial pull force	5
main pull force	25
main pull delay	8
heater control	320

The micropipette was held vertically, a 1 µl aliquot of DNA was dispensed at the top and allowed to reach the needle tip by capillary action. When ready to inject the sealed tip was broken back by a slight tap on the edge of the slide.

2.13.3 Microinjection

The embryos were injected with a mixture of pP{UAST} (Brand and Perrimon, 1993) or pP{CaSpeR.hs/act} (Thummel *et al*, 1988) containing the sequence of interest (400 ng) and the helper plasmid pP{Δ2-3} (100 ng), which had been diluted in injection buffer (0.1 mM Na_2HPO_4, 0.1 mM NaH_2PO_4, 5 mM KCl, pH 7.8). Embryos were injected with this solution using an Eppendorf Micromanipulator InjectMan connected to an Eppendorf FemtoJet® pressure modulator. Embryos were viewed under a Zeiss inverted stage microscope.

2.13.4 Post-injection care

Slides containing injected embryos were placed on a fresh grape-juice agar plate. The embryos were left to recover for 1-7 d at 18°C and any hatched larvae transferred to standard food. Surviving adults were mated individually back to the host strain and the progeny scored for eye colour transformation. Transformants were crossed again to the host strain and transformed progeny of this cross were mated to siblings. Selection against flies with white eyes through generations allowed, in most cases, homozygous lines to be generated for the P-element insertion.

2.14 Cyclic AMP assay

This protocol is an adaption of the Biotrak cAMP [^{125}I] assay system (Amersham), as previously described in Davies *et al*, 1995. The assay is based on the competition between unlabelled cAMP and a fixed quantity of ^{125}I-labelled cAMP for a limited number of sites on a cAMP-specific antibody. With fixed amounts of antibody and radiolabelled cAMP, the amount of radiolabelled cAMP bound by the antibody will be inversely proportional to the concentration of non-radiolabelled cAMP contained in the tissue homogenate. The antibody bound cAMP is then reacted with a second antibody which is bound to magnetizable polymer particles. Separation of the antibody bound fraction is achieved by centrifugation and aspiration of the supernatant. Measurement of the radioactivity in the pellet enables the amount of radiolabelled cAMP in the bound fraction to be calculated. The amount of unlabelled cAMP in the sample is then determined by interpolation from a standard curve.

2.14.1 Preparation of samples

Tubules were dissected in Schneider's medium (Gibco BRL). Ten pairs were transferred to 2 ml collection tubes containing 100µl Schneider's medium. Samples were prepared in triplicate and incubated for 20 min with the non-specific phosphodiesterase inhibitor IBMX ($1x10^{-5}$ M) and, if necessary, the appropriate antagonist. When required, agonist was added at varied concentrations after 10 min. All reactions were quenched with 70% ice cold ethanol after 20 min and transferred to ice. The tubules were then homogenised for 1 min using a Polytron homogeniser (Kinematica), and vacuum dried for several hours. Dried samples were stored at –70°C until required.

2.14.2 Resuspension of samples

Samples were resuspended in 500 µl assay buffer (0.05 M sodium acetate, pH 5.8 containing sodium azide).

2.14.3 Cyclic AMP assay procedure

Serial dilutions of a cAMP standard (32 pmol/ml) were prepared, in duplicate, ranging from 25-1600 fmol to generate a standard curve against which samples of unknown cAMP content could be compared. Duplicate zero standard tubes were prepared containing assay buffer only. For use in the assay, 100 µl aliquots were taken from all standard and unknown tubes and dispensed into appropriately labelled reaction tubes. Into all tubes 100 µl of [^{125}I] cAMP was added, followed by 100 µl of antiserum. A tube containing only 100 µl of [^{125}I] cAMP was prepared as a measure of the total amount of radioactivity available per reaction. This tube was labelled "total count" (TC).

The tubes were then thoroughly vortex mixed, wrapped in lead and incubated for 3 hs at 4°C. After incubation, 500 µl of second antibody reagent was added to all tubes, except the total count tube, vortex mixed immediately, and incubated for 10 min at room temperature. The antibody bound fraction was separated by centrifugation for 10 min at 13 000 rpm. The supernatant was carefully aspirated and tubes were placed in a gamma scintillation counter for 1 min.

2.14.4 Calculation of cAMP content

The average counts per min (cpm) were calculated for the duplicate standards and zero standards. The percent of the total count [^{125}I] cAMP bound to second antibody in the zero standard (B_0) was calculated as an indicator of antibody efficacy using the following equation:

$$\%/B_0 / TC = \frac{B_0 \text{ cpm}}{TC \text{ cpm}}$$

The percent bound [^{125}I] cAMP for each standard and sample was calculated using the following equation:

$$\%B/B_0 = \frac{(\text{Standard or sample cpm})}{B_0 \text{ cpm}}$$

Standard curves were generated by plotting the percent B/B_0 as a function of the log cAMP concentration. B/B_0 was plotted on the y-axis against the fmol standard per tube on the x-axis. The fmol per tube value for samples could then be read directly from the graph. Figure 2.2 shows a typical standard curve generated in this assay. This value was then divided by the number of tubules in each sample (20) to obtain a per tubule value.

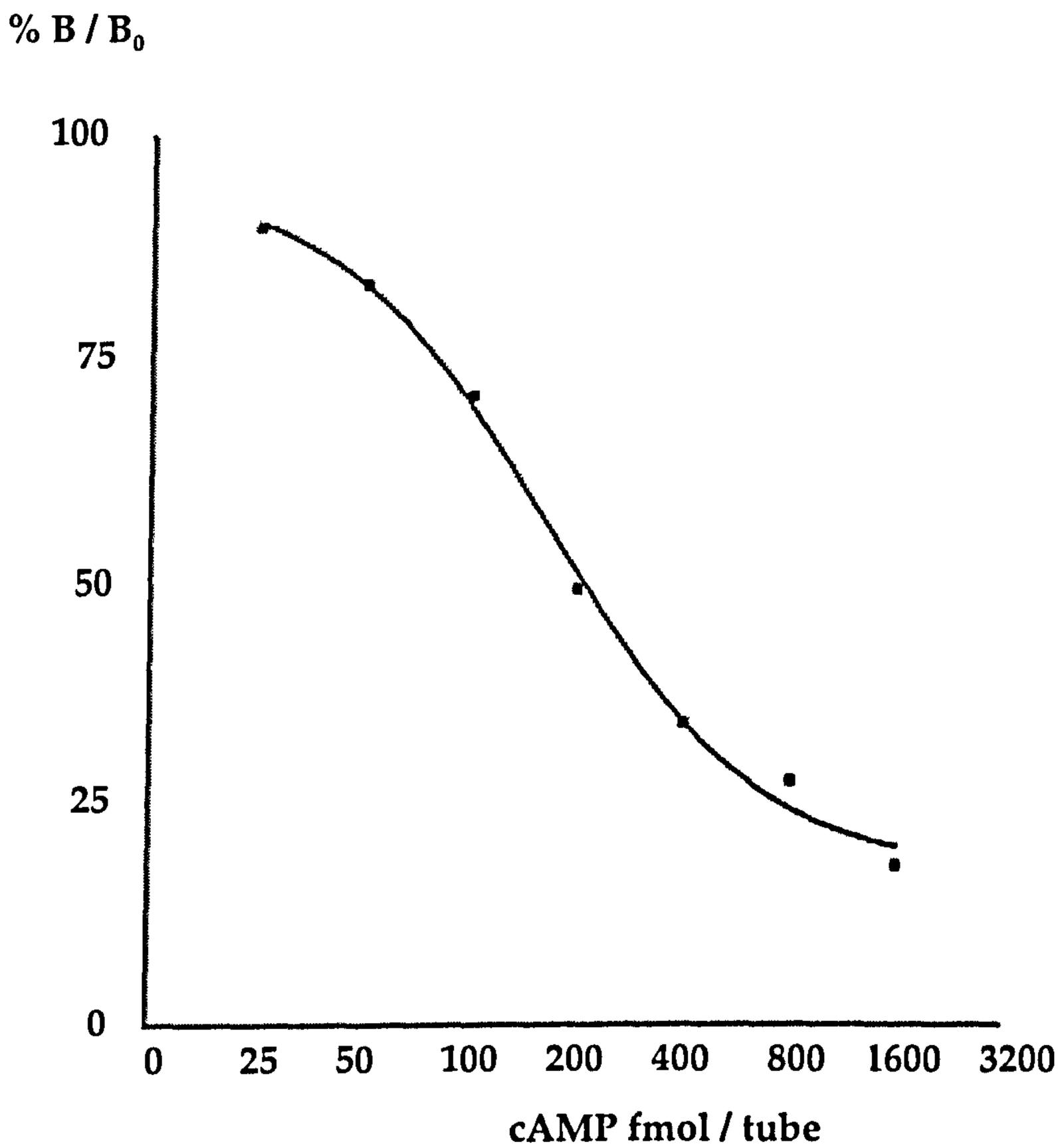

Figure 2.2: A typical standard curve generated for a cAMP assay

2.15 Cyclic GMP assay

The general principles of this assay are the same as those for the cyclic AMP assay and have been described previously in Dow *et al* 1994a. The assay is based on the competition of radiolabelled cGMP with endogenous tissue cGMP for a limited number of sites on a cGMP-specific antibody. With fixed amounts of antibody and radioactive ligand, the amount of radioactive ligand bound by the antibody will be inversely proportional to the concentration of added non-radioactive ligand. However, cGMP in samples and standards was acetylated at the 2'0 position by acetic anhydride. 2'0 substituted cyclic nucleotides have greater affinity for the antibody than parent cyclic nucleotides because the antibody is made to a protein conjugate coupled at the 2'0 position, thus making the assay more sensitive (Harper and Brooker, 1975). This is necessary, as intracellular cGMP levels are greater than 10 fold lower than intracellular cAMP levels in tubules (Davies *et al*, 1995).

2.15.1 Preparation of samples

Samples were prepared as in the cAMP assay, but with an initial incubation in the cGMP-specific phosphodiesterase inhibitor zaprinast (1×10^{-6} M) rather than with IBMX.

2.15.2 Resuspension of samples

Samples were resuspended in 500 µl assay buffer (0.05 M sodium acetate, pH 5.8 containing sodium azide).

2.15.3 Cyclic GMP assay procedure

Serial dilutions of a cGMP standard (2.56 pmol/ml) were prepared, in duplicate, ranging from 2-128 fmol. Duplicate zero standard tubes were prepared containing 500 µl assay buffer only. An acetylation reagent was prepared by mixing 1 volume of acetic anhydride with 2 volumes acetic anhydride in a glass vessel. 25 µl of acetylation reagent was added to all tubes containing standards and unknowns and 100 µl aliquots were transferred to appropriately labelled reaction tubes. 100 µl of antiserum was then added to all tubes and were vortex mixed immediately. Tubes were covered and incubated at room temperature for 1 h before addition of 100 µl of [^{125}I] cGMP. A 100 µl aliquot of [^{125}I] cGMP was also added to an empty "total count" tube. Tubes were vortex mixed again, wrapped in lead and incubated for a further 18 h at 4°C.

The following day 500 µl of second antibody bound to beads was added to all tubes, except the total count tube. Tubes were vortex mixed and incubated at room temperature for 10 min before separation of the antibody bound fraction by centrifugation (13 000 rpm for 10 min). The supernatant was then decanted and radioactivity present in each tube was determined by counting for 60 s in a gamma scintillation counter.

2.15.4 Calculation of cGMP content

Calculation of cGMP content in samples was determined in exactly the same manner as with the cAMP assay. An example of a typical standard curve generated in this assay is shown in Figure 2.3.

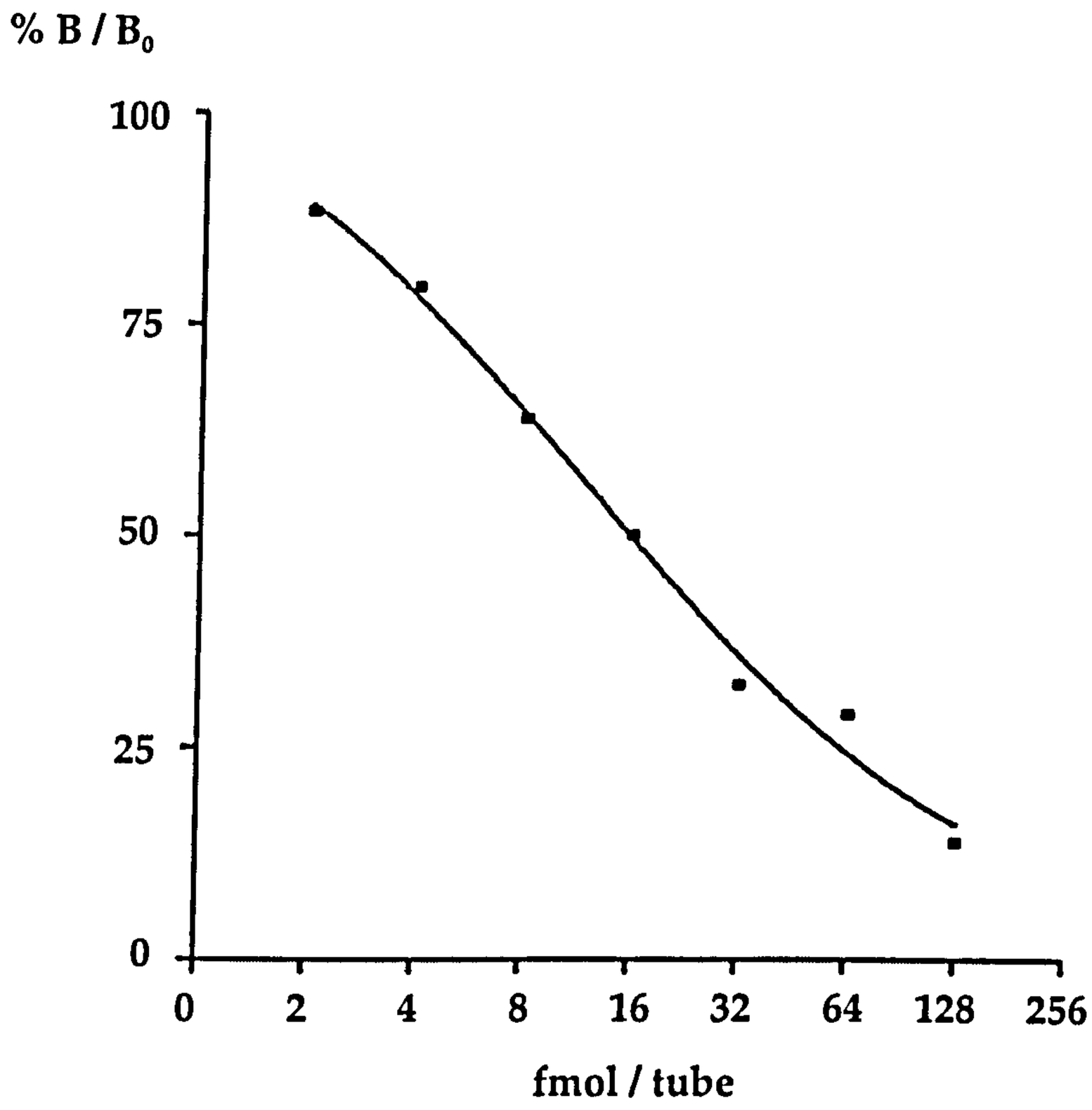

Figure 2.3: A typical standard curve generated in a cGMP assay

2.16 Western Blot analyses

For Western analyses, tissues were homogenised in 130 µl of Tris-lysis buffer (2% SDS, 70 mM Tris pH 6.8) + 1 µl protease inhibitor cocktail (Calbiochem, 500 µM AEBSF, 500 µM EDTA, 1 µM E-64, 1 µM leupeptin and 1 mg/ml Aprotinin) and centrifuged to remove debris.

The protein concentration of each sample was measured using the Lowry protein assay. To each 25 µl of unknown or standard protein sample 100 µl of Folin & Ciocalteu's phenol reagent (Sigma) and, 1 ml of solution X (10 µl 1% $CuSO_4$, 10 µl 2% K^+Na^+ tartate, 1ml 10% Na_2CO_3 in 0.1 M NaOH) was added. Absorbance of each sample was then measured at 750 nM with a spectrophotometer and unknown sample concentrations estimated through comparison with known 1-10 mg/ml BSA standards. The samples were then electrophoresed on a 3-8 % Tris-acetate gel with 5 µl loading buffer (60 % glycerol, 12.5 % β-mercaptoethanol, 1 % bromophenol blue) for 60 min at 100 V in running buffer (see Appendix 4). A nitrocellulose membrane was cut to the size of the gel and soaked for 10 min in H_2O, then 10 min in transfer buffer. The blot for transfer was prepared according to kit instructions (NuPage Electrophoresis system, NOVEX), and the proteins were transferred from gel to membrane by electric current (45 min at 190 V).

Transfer of proteins was affirmed by staining the membrane with Ponceau dye. The membrane was then washed twice in distilled water, before being blocked in 5 % dehydrated milk powder, 0.1 % Tween in 1 X PBS for 1-2 h. The membrane was hybridised with the appropriate primary antibody diluted in 5% dehydrated milk powder, 0.1 % Tween in 1 X PBS overnight. After two 30 min washes in 1 X PBS, the membrane was hybridised with the secondary antibody diluted in 1 X PBS.

2.17 Immunocytochemistry

This protocol is the same as described in MacPherson *et al*, 2001. Slides were washed with water and dried with ethanol, before a wax circle was drawn on using a wax pen (Vector). Slides were treated with 100 µl 0.1 mg/ml poly-L-lysine solution (Sigma) for 30 min, then washed with tap water and left to air dry. Tubules were dissected in Schneider's *Drosophila* medium and stuck onto slides in 1 X PBS solution. Tubules were fixed with 4% paraformaldehyde in 1 X PBS for 30 min, then washed 5-7 times in 1 X PBS before permeabilisation with 0.2% (v/v) Triton X-100 in PBS for 30 min. Permeabilisation solution was changed every ten min, after which the tubules were blocked for 3 h in PAT (PBS containing 0.5% (w/v) Sigma cold fraction V bovine serum albumin and 0.2% (v/v) Triton X-100). Samples were then hybridised overnight in a humidity chamber with the primary antibody diluted in PAT. The following day, slides were washed four times in PAT over a period of 2 h. They were then blocked with PAT containing 2% (v/v) normal goat serum (Scottish Antibody Production Unit (SAPU)) for a further 4 h. The samples were next incubated overnight with the secondary antibody diluted in PAT with 2% normal goat serum in a humidity chamber. The slides were then washed four times over a period of 2 h in PAT and twice for 10 min in PBS before being mounted in Vectashield mounting medium (Vector). Slides were examined under UV light with a conventional light microscope (Zeiss).

2.18 Antibodies

Antibody	Raised in	1° or 2°	Epitope	Use	Specificity	Dilution
R1214 anti-GC-A (Kind gift, David Garbers)	Rabbit	1°	Glu-Arg-Gly-Cys-Ser-Th-Arg-Gly	Immunocytochemistry Western blot	N/A N/A	1:7000 1:250
Anti-cGMP polyclonal antiserum (Chemicon Int. Inc. AB303)	Rabbit	1°	2'-O-succinyl cGMP-BSA	Immunocytochemistry	cGMP: 100% No cross reaction to other nucleotides at concentrations to 1 mM	1:7000
Anti-cAMP polyclonal antiserum (US Biological)	Rabbit	1°	2'-O-succinyl cAMP-BSA	Immunocytochemistry	cAMP: 100% ATP: ≤ 0.01% cGMP: 0.1% 2'3'AMP:≤ 0.01% cIMP: 0.1% cCMP: 0.07% ADP: ≤ 0.01%	1:200
Anti-rabbit-fluorescein	Donkey	2°	Rabbit IgG	Immunocytochemistry	N/A	1:250

Table 2.3: Antibodies used in this study

2.19 Cyclic GMP-dependent kinase assay

This protocol has been adapted from the SignaTECT™ cyclic AMP-dependent protein kinase assay system (Promega) (M.MacPherson, unpublished) and is similar to that described in Osborne *et al*, 1997. The method involves measuring the transfer of ^{32}P-labelled phosphate to a peptide substrate that is captured on phosphocellulose filter via weak electrostatic interactions. However, in the presence of multiple kinases in a tissue extract, the ^{32}P-labelled peptides/proteins bound to the phosphocellulose filter may reflect kinase activity other than that due to cGMP-dependent protein kinases (cGKs). To increase the specificity of the cGK assay the heptapeptide glasstide (RKRSRAE, Calbiochem), a specific substrate for cGK, was used in the reaction (Hall *et al*, 1999). The inclusion of PKA inhibitor (Sigma) in the reaction also helped minimize background phosphorylation.

2.19.1 Sample preparation

Approximately 800 tubules were dissected and placed in 200 µl buffer (20 mM Tris, 150 mM sucrose, 2 mM EDTA, 100 mM NaCl, 50 mM β-mercaptoethanol, 2 µg/ml leupeptin, 5 µg/ml aprotinin, 1 µg/ml PMSF). Appropriate samples were treated with agonist for 10 min before being homogenised and centrifuged for 5 min at 13 000 rpm. A Lowry protein assay was then conducted to determine the protein concentration of samples, and samples were diluted to equivalent concentrations. Approximately 200 ng protein was used for each experimental condition.

Two reaction mixes were prepared with and without addition of cGMP (20 mM Tris, 20 mM MgAc, 100 µM zaprinast, 100 µg/ml glasstide (RKRSRAE), 200 µM ATP, 2 µl [γ-^{32}P] ATP (10 µCi/µl), 1 nM PKA inhibitor, +/- 100 µl cGMP (10 µM) in 1 ml).

40 µl reaction buffer was added to 5 µl protein sample. For each protein preparation, separate conditions with and without cGMP were set up using the two different reaction mixes. Reactions were incubated for 30 min at 30 °C, after which 25 µl of each sample was spotted onto individual squares of P81 paper (Whatman). These squares of paper are referred to as reaction samples. Several samples were chosen randomly and 5 µl aliquots spotted onto individual squares of P81 paper, allowed to dry and set aside. These samples (representative of 1/9 th of total count) are necessary to determine the specific activity of the radiolabelled ATP.

The reaction squares were washed 3 times for 5 min in 75 mM phosphoric acid, then washed once for 15-20 sec in ethanol and allowed to dry. All squares of paper, including the specific activity samples were then transferred to scintillation vials and covered with 3 ml scintillation fluid. Radioactive counts from were measured in a scintillation counter for 60 sec.

2.19.2 Calculation of kinase activity

Specific activity of [γ-^{32}P]ATP was calculated using the following equation :

$$\text{Specific activity} = \frac{\text{(Total reaction vol/5) x Average activity of 5µl total count squares}}{\text{Total conc ATP used in reaction}}$$

Units of specific activity = cpm/pmol ATP

Protein kinase enzyme activity was calculated using the following equation :

$$\frac{\text{(cpm of reaction sample - cpm of blank sample)}}{\text{vol sample on filter x time x amt protein in sample x specific activity}}$$

Units of enzyme activity = pmol ATP/min/µg protein.

2.20 Phosphodiesterase Assay

The radioactive assay of cyclic nucleotide phosphodiesterase activity was first described by Thompson and Appleman (Thompson and Appleman, 1971; Thompson *et al*, 1974). The protocol for measurement of PDE activity in *Drosophila* Malpighian tubules was developed by Kate Broderick (manuscript in preparation) and assays were performed in the laboratory of Prof. N. Pyne, University of Strathclyde. Use of radioactive tracers (tritiated cAMP or cGMP) allows enzyme activity to be measured at subsaturating levels of substrate. Measurement of enzyme activity involves quantification of reaction product formed. A second incubation step converts the product of the first incubation (5'-nucleotide monophosphate) to 5'-nucleotide, which is catalysed by 5'-nucleosidase present in snake venom. This allows the reaction product to be more effectively separated from the unreacted substrate.

The two incubation steps can be represented thus:

(i) 3',5'[8-^{3}H]-cyclic NMP → 5'[8-^{3}H]-NMP

(ii) 5'[8-^{3}H]-NMP → [8-^{3}H]-nucleoside

Step (i) is catalysed by cyclic NMP (cNMP) phosphodiesterase; Step (ii) catalysed by 5'-nucleotidase; NMP = nucleotide monophosphate.

Separation uses Dowex-1-chloride anion exchange resin which selectively binds cyclic nucleotide. Quantification is by liquid scintillation counting of the isolated product (tritiated 5'-nucleotide).

2.20.1 Preparaton of samples

200 tubules (80-120 μg of protein) were dissected into 150 μl of PDE buffer and agonist added to appropriate samples for 10 min before being homogenised. 50 μl of ^{3}H cGMP working stock (0.185 kBq/μl in 1 μM cGMP, 10 mM Tris, 5 mM $MgCl_2$, pH7.4.) was added to a 50 μl aliquot of each sample. Blank samples were prepared containing 50 μl of PBS and 50 μl of working stock ^{3}H cGMP. Positive controls were made by preparing samples with 50 μl TOPO TOP10 cell lysates transformed with pcDNA3.1-PDE5A1 and 50 μl of working stock ^{3}H cGMP.

The same procedure was followed for cAMP-dependent phosphodiesterase assays, but with ^{3}H cAMP in place of ^{3}H cGMP and TOP10 cell lysates transformed with pcDNA3.1-PDE4 as a positive control.

2.20.2 Assay procedure

The samples were incubated at 30°C for 10 min and then boiled for 2 min to stop the reaction. The samples were cooled on ice and 25 μl of 1 mg/ml snake venom added and incubated for 10 min. 400 μl of re-suspended Dowex-1-chloride (Sigma) was then added and the mix was vortex mixed every 5 min for 15 min. The samples were centrifuged for 2 min at 12 000 rpm. 150 μl of the supernatant was then removed and added to 2 ml scintillation fluid. The samples were counted in a scintillation counter for 60 s.

2.20.3 Calculation of PDE activity

Enzyme activity was calculated using the following equation:

$$\frac{\text{cpm of sample x total assay vol x nucleoside binding factor x pmol cNMP}}{\text{Total cpm x assay vol measured x incubation time x protein conc}}$$

Units are in pmol/min/mg protein.

2.21 $[Ca^{2+}]_i$ measurements in aequorin expressing tubules.

The method for measurement of $[Ca^{2+}]_i$ in aequorin expressing tubules is described in Rosay *et al*, 1997, and is as follows. For reconstitution of intracellular aequorin, 50 tubules from 3-7 day old adults were dissected in Schneider's medium and placed in 160 µl of Schneider's medium with coelentrazine, to a final concentration of 2.5 µM. Samples were then incubated for 3 h in darkness. Bioluminescence recordings were carried out using an LB9507 luminometer (Berthold Wallac). Samples were 'mock' injected with 20 µl of Schneider's before injection with 20 µl of appropriate agonist at 10 X the desired concentration. At the end of each recording tubules were disrupted in 350 µl lysis solution (1% (v/v) Triton X-100, 100 mM $CaCl_2$, causing discharge of the remaining aequorin, thus allowing an estimation of the total amount of aequorin in the sample by integration of total counts. Calcium concentrations for each time point in an experiment were calculated by backward integration, using a program written in Perl, based on the method described by Button and Eidsath (1996).

Chapter 3

GC-A
&
cGMP signalling

3.1 Summary

A rat receptor guanylate cyclase gene (GC-A) was chosen as a tool to specifically modulate cGMP in tubules. This receptor met two prerequisites: firstly, the receptor directly activates cGMP production upon activation, and secondly, the ligand to this receptor, ANP, does not elicit a response in wild-type tubules.

A 3.1 kb open reading frame was subcloned into the P-element vectors pP{CaSpeRhs/act} and pP{UAST} to allow temporal and spatial control of expression of the gene, respectively. The insertion of the construct into the genome of individual lines of flies was confirmed by PCR and the locus identified by inverse PCR. Transcription of the gene was detected in tubules by reverse transcriptase PCR. Western blots revealed a protein product of 180 kDa, consistent with other findings (Paul *et al*, 1989; Koller *et al*, 1992) and immunocytochemical studies localised the protein to the basolateral membrane of tubules.

Fluid secretion was elevated in tubules expressing GC-A throughout tubules, or in a cell-specific manner, in response to ANP. This response was dose-dependent, and transgenic tubules treated with very high concentrations (>1 mM) ANP showed an attenuation of fluid secretion. The GC-A specific antagonist anantin attenuated the response seen in transgenic tubules to ANP.

Intracellular cGMP content was measured by radioimmunoassay in transgenic tubules, and shown to be elevated in response to ANP in a dose dependent manner. This could be attenuated with anantin, consistent with fluid secretion data. Using an antibody to cGMP, production of the second messenger was detected immunocytochemically in tubules that were challenged with ANP, whereas background levels were shown to be very low.

The specificity of cGMP as a downstream effector of GC-A activation was determined by measuring intracellular cAMP levels in ANP-stimulated tubules.

No significant change in intracellular cAMP levels between stimulated and unstimulated samples were observed by radioimmunoassay, suggesting that GC-A acts specifically to generate cGMP.

The possibility of elevation of intracellular cGMP affecting Ca^{2+} levels as a result of cross-talk was investigated by aequorin bioluminescence assay. A biphasic elevation of intracellular Ca^{2+} was observed when GC-A was expressed in principal cells, suggesting that there is a degree of cross-talk between the cGMP pathway and Ca^{2+} signalling. However, no effect on Ca^{2+} levels was seen when GC-A was expressed in stellate cells only, which suggests a differential response to cGMP in different cell types.

Tubules have previously been shown to express two cGMP-dependent kinases (cGK), namely *dg1* and *dg2* (Dow *et al*, 1994a) and the activity of these kinases is likely to alter as a consequence of intracellular cGMP production. Cyclic GMP-dependent kinase activity was therefore assayed in tubules expressing GC-A in a cell-specific manner in the presence and absence of ANP. In order to determine ANP-induced changes in kinase activity, as opposed to absolute kinase activity, tubule samples were prepared in assay buffer lacking cGMP. This ensured that kinase activity measured was due directly to endogenous intracellular cGMP generated by GC-A. Kinase activity was elevated upon incubation with ANP in tubules expressing GC-A cell-specifically in either principal or stellate cells.

Interestingly, when the assay was carried out in assay buffer containing 10 μM cGMP, there was not a consistent absolute kinase activity amongst all samples; samples treated with ANP had a potentiated kinase activity.

In most signalling systems, there is the requirement for a negative feedback loop to allow a signal to be dampened, or turned off. The only known enzymatic reaction for the degradation of cyclic nucleotide is its conversion to 5′-nucleoside by cyclic nucleotide phosphodiesterases. Therefore, activity of

cGMP- and cAMP-dependent phosphodiesterases in GC-A transgenic tubules was assayed, both in the presence and absence of ANP. There was a marked increase in cGMP-dependent phosphodiesterase activity in ANP-stimulated tubules, indicating a negative feedback mechanism. Interestingly, there was a slight decrease in cAMP-dependent phosphodiesterase activity in response to ANP, although cAMP levels remained unaltered. This might potentiate a cAMP signal only when adenylate cyclase activity is elevated.

Finally, the use of GC-A as a generic tool for manipulating cGMP in any tissue was illustrated by the elevation of intracellular levels of cGMP in brains taken from heat-shocked hs::GC-A flies, in the presence of ANP.

These results directly demonstrate that cGMP is a primary modulator of fluid transport in Malpighian tubules and that activation of GC-A by ANP leads to both elevated cGMP-dependent kinase and phosphodiesterase activity. Furthermore, there is evidence for either cross-talk between cGMP- and cAMP-dependent phosphodiesterases or elevation of a dual specificity phosphodiesterase by cGMP. In principal cells, the direct elevation of cGMP in response to ANP, leads to an elevation of intracellular Ca^{2+} levels. This link to Ca^{2+} signalling is not present in stellate cells, which unravels differential cellular responses to elevated intracellular cGMP.

This work demonstrates that ectopic expression of GC-A in *Drosophila* can therefore be used as a tool for manipulating intracellular cGMP *in vivo*.

3.2 Introduction

There are a number of putative roles for intracellular cGMP. It has been implicated in direct ion channel activation, and several cGMP dependent protein kinases and phosphodiesterases have been identified. Overall, cGMP can influence cell function through protein phosphorylation or mechanisms not directly related to phosphorylation. It controls diverse physiological responses, such as vascular smooth muscle relaxation and neutrophil activation, as well as having an essential role in normal kidney function (Lopez *et al*, 1995; Dubois *et al*, 2000). It has also been suggested that cGMP pools generated at the plasma membrane by membrane guanylate cyclases have a role in control of intracellular Ca^{2+} levels (Zolle *et al*, 2000).

Fluid transport by Malpighian tubules has been shown to be controlled, in part, by elevation of cGMP via activation of a soluble guanylate cyclase (Dow *et al*, 1994a; Davies *et al*, 1995). However, the distinct role of cGMP may be difficult to infer when levels are elevated by activation of the endogenous receptor for the neuropeptide CAP_{2b} (Davies *et al*, 1995). This receptor activates cGMP/NO signalling via a calcium/calmodulin dependent nitric oxide synthase (*dNOS*); this process is also dependent on calcium influx to the tubule (Rosay *et al*, 1997; MacPherson *et al*, 2001; MacPherson *et al*, in preparation). There is therefore likely to be a bifurcation of signals when Ca^{2+} levels are altered by this receptor, upstream of guanylate cyclase activation. An approach whereby the role of cGMP can be studied without the complications created by use of an endogenous peptide is therefore required. To achieve this, a gene encoding a guanylate cyclase that, upon activation, directly stimulates cGMP was ectopically expressed in tubules. The rat GC-A receptor was chosen for this study, as tubules have been shown not to respond to its ligand, atrial natriuretic peptide (ANP) under normal conditions (Dow *et al*, 1994a). Unlike GPCRs, this receptor directly affects second messenger levels through its guanylate cyclase

domain. This system thus does not impose any requirement for the expression of cognate G proteins in the host cell. Furthermore, there are no ANP-like peptides encoded in the *Drosophila* genome (data not shown), making GC-A a generally useful receptor to study cGMP signalling in this organism.

3.2.1 Guanylate cyclases

Guanylate cyclases are a family of enzymes that catalyze the formation of cGMP from the purine nucleotide GTP. The family comprises both membrane-bound and soluble isoforms that are expressed in many cell types. They are regulated by diverse extracellular agonists, including peptide hormones, bacterial toxins, and free radicals, as well as intracellular molecules, such as calcium and adenine nucleotides.

Guanylate cyclases are divided into two classes; a soluble haem containing heterodimer, and a single-pass transmembrane form, which forms homodimers, or even homotetramers (for review, see Lucas *et al*, 2000). The ligands for the soluble guanylate cyclases are NO (Gerzer *et al*, 1981a; Gerzer *et al*, 1981b; Drewitt and Garbers, 1994; Garbers *et al*, 1994) and CO (Snyder and Bredt, 1992; Friebe *et al*, 1996), which bind to the prosthetic haem group. The ligands for the membrane guanylate cyclases fall into two categories, namely natriuretic peptides (NPs) and the heat-stable enterotoxins of *E.coli*, for which the endogenous homologues are guanylin/uroguanylin. In mammals there are at least six forms of membrane guanylate cyclase, named GC-A-F (Garbers, 1992), and ligands from the natriuretic peptide (NP) family have been identified for three (GC-A-C): GC-A is activated by atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) at physiologic concentrations, but not C-type natriuretic peptide (CNP). Conversely, CNP, but not ANP or BNP, activates GC-B. In addition, all three natriuretic peptides bind to the natriuretic peptide clearance receptor, GC-C, with similar affinities (Maack, 1992). This receptor has no guanylate cyclase activity and is thought to regulate concentrations of

NP available to bind to GC-A and GC-B (Maack, 1992). However, a signalling function for this receptor has also been reported (Anand-Srivastava and Trachte, 1993), altering cell function through G_{i-2} protein-coupled inhibition of membrane-bound adenylate cyclase activity (Murthy and Makhlouf, 1999; Murthy and Makhlouf, 2000; Yanaka *et al*, 1998) or activation of phospholipase C via the βγ-subunits of G_{i-1} and G_{i-2} (Murthy *et al*, 1999).

In this study, we chose to use ANP, the ligand with highest affinity for GC-A. ANP was originally identified in atrial myocardial extracts as a factor promoting natriuresis (de Bold *et al*, 1981) and contains a 17-amino acid disulphide ring with a highly conserved sequence (FGXXXDRIGXXSGL) shared amongst all natriuretic peptides. Similarly, anantin, a peptide isolated from a strain of *Streptomyces coerulescens*, consists of 17 amino acids which from a peptidic ring, and binds to GC-A (Weber *et al*, 1991). This peptide does not activate the receptor, and acts as a competitive antagonist of GC-A.

Work described in this chapter aims to look at the direct role of cGMP in *Drosophila* Malpighian tubules using the rat GC-A receptor as a modulator of intracellular cGMP levels.

3.3 Results

3.3.1 Cloning of GC-A into P-element vectors pP{CaSpeRhs/act} and pP{UAST}

A rat particulate guanylate cyclase (GC-A) was provided as a 3.7 kb cDNA with the upstream ATGs removed, cloned into the *Eco* RI site of pBluescript KS+ (Chinkers *et al*, 1989, kind gift of Michael Chinkers, University of Alabama).

A 3.1 kb fragment containing the open reading frame with engineered 5' *Bgl* II and 3' *Not* I restriction sites was amplified by PCR, using a combination of *Taq* and *Pwo* DNA polymerases. The PCR product was subcloned into the TOPO TA cloning vector pCR2.1 (Invitrogen) to allow efficient digestion at the 5' and 3' ends with *Bgl* II and *Not* I restriction enzymes, respectively. The cloned fragment was sequenced before being cloned into the 5' *Bgl* II and 3' *Not* I restriction sites in pP{UAST} (Figure 3.1) and pP{CaSpeRhs/act} (Figure 3.2) and then sequenced again to confirm error free directional cloning of the open reading frame. Transgenic lines created with these constructs are referred to as hs::GC-A and UAS::GC-A respectively.

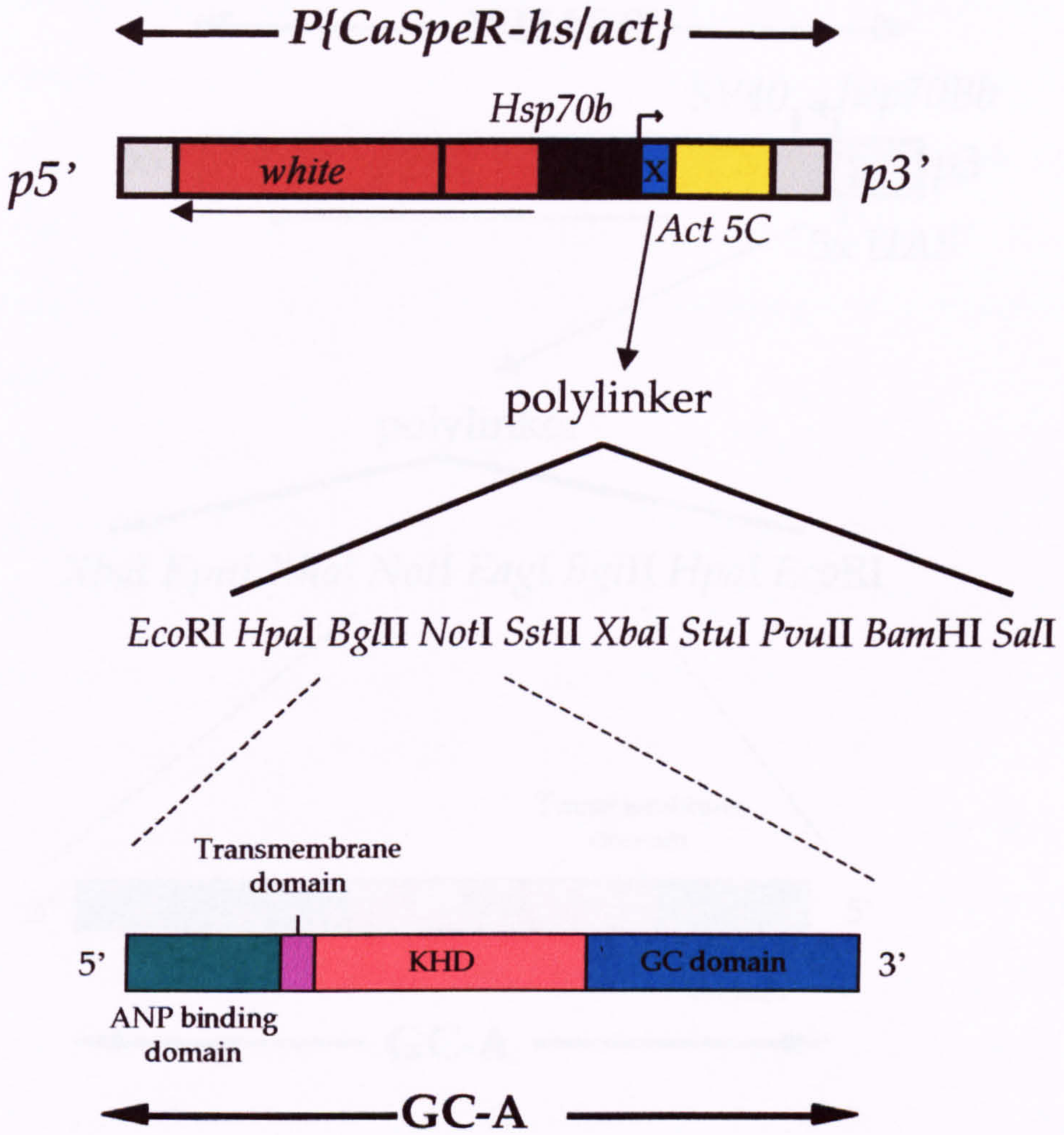

Figure 3.1: Cloning of rat GC-A into pP{CaSpeR-hs/act} vector

A 3.1 kb rat GC-A open reading frame was amplified from a cDNA clone in pBluescript KS+ (kind gift, Michael Chinkers). Primers used (Appendix 1) introduced 5' *Bgl* II and 3' *Not* I to the open reading frame to allow for directional cloning into pP{CaSpeRhs/act} (Thummel *et al*, 1988).

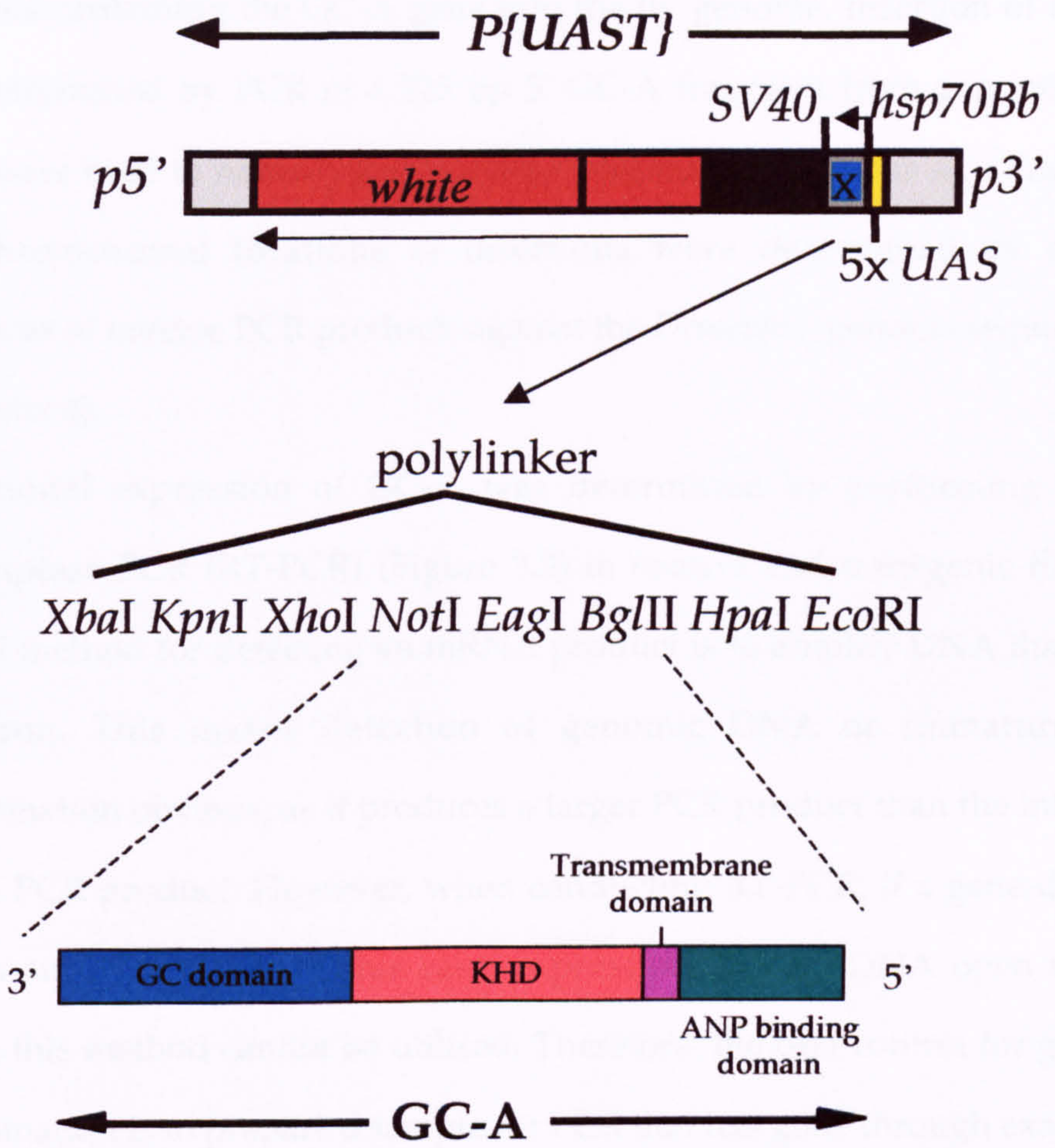

Figure 3.2: Cloning of rat GC-A into pP{UAST} vector

A 3.1 kb rat GC-A open reading frame was amplified from a cDNA clone in pBluescript KS+ (kind gift, Michael Chinkers). Primers used introduced (Appendix 1) 5' *Bgl* II and 3' *Not* I to the open reading frame to allow for directional cloning into pP{UAST} (Brand and Perrimon, 1993).

3.3.2 Expression of GC-A in *Drosophila melanogaster.*

Transgenic lines were generated by random insertion of the engineered P-elements containing the GC-A gene into the fly genome. Insertion of the gene was determined by PCR of a 525 bp 5′ GC-A fragment from genomic DNA. Lines were bred to homozygosity where possible, and kept as separate stocks. The chromosomal locations of insertions were determined by aligning sequences of inverse PCR products against the *Drosophila* genome sequence (see Appendix 4).

Conditional expression of GC-A was determined by performing reverse transcriptase PCR (RT-PCR) (Figure 3.3) in control and transgenic flies. The general method for detecting an mRNA product is to amplify DNA that flanks an intron. This makes detection of genomic DNA or immature RNA contamination obvious, as it produces a larger PCR product than the intronless mRNA PCR product. However, when conducting RT-PCR, if a gene does not contain any introns (or in this case, expression is of a cDNA open reading frame), this method cannot be utilised. Therefore, the best control for genomic contamination is to prepare a sample for PCR that has gone through exactly the same procedure for generation of cDNA template as all other samples, with the omission of reverse transcriptase enzyme. Any PCR product obtained from preparations like this would then represent contamination. An additional control PCR was conducted using intron flanking primers for a product known to be present in tubules (protein kinase C (PKC)). The absence of genomic product when cDNA was used as PCR template further confirmed no genomic contamination.

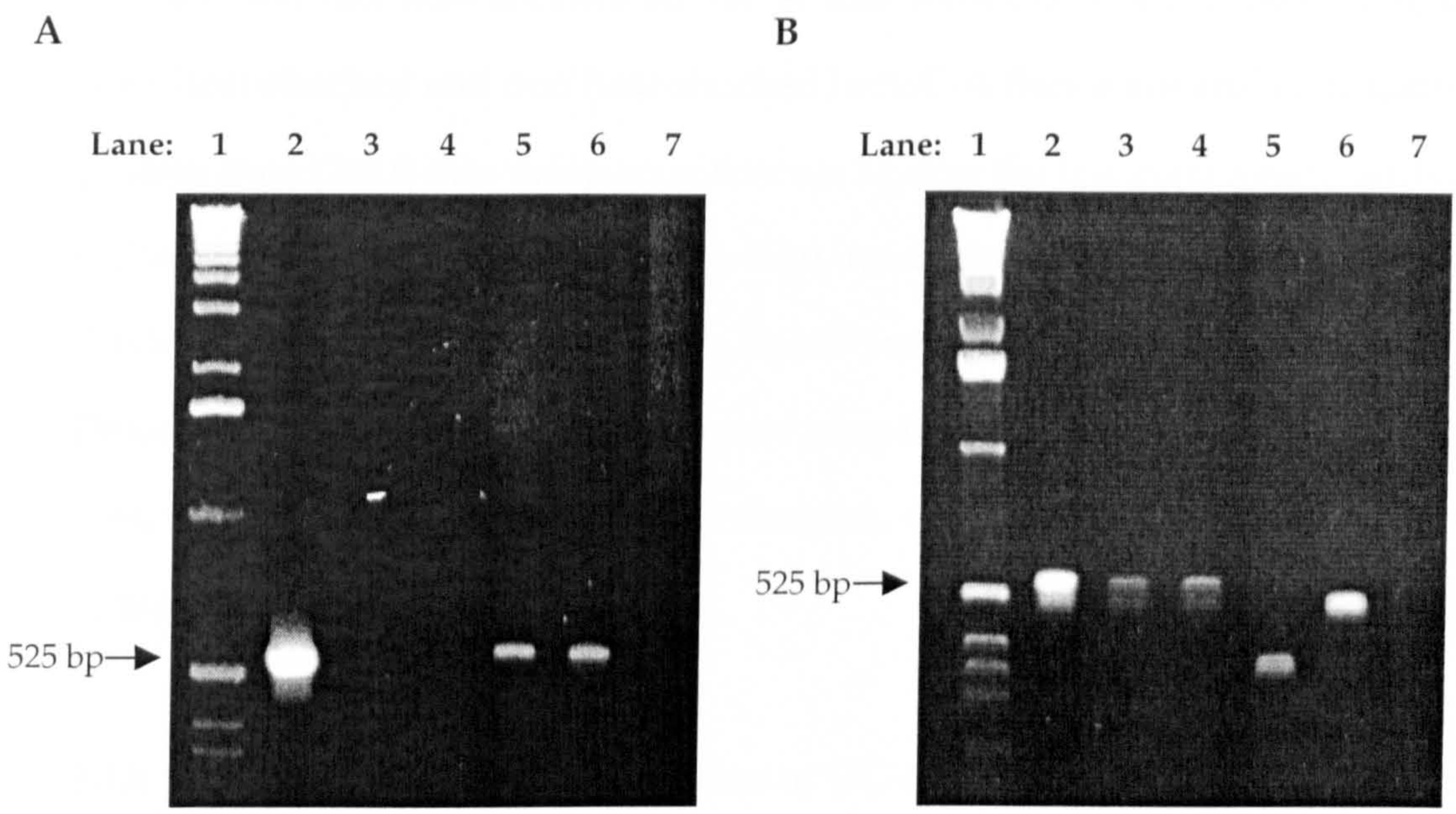

Figure 3.3: The expression of GC-A in transgenic lines

Reverse-transcription polymerase chain reaction (RT-PCR).

A: Lane 1: 1kb ladder (Gibco BRL). Lane 2: PCR using *Drosophila melanogaster* genomic DNA from pP{CaSpeRhs::GC-A} flies as a positive control. Lane 3: cDNA template prepared from non heat-shocked pP{CaSpeRhs::GC-A} flies. Lane 4: cDNA (no superscript) prepared from heat-shocked pP{CaSpeRhs::GC-A} flies. Lanes 5 & 6: cDNAs prepared from heat-shocked pP{CaSpeRhs::GC-A} flies. Lane 7: PCR using wild-type genomic DNA as a negative control.

B: Lane 1: 1kb ladder (Gibco BRL). Lane 2: cDNA template prepared from c42/UAS::GC-A tubules. Lane 3: cDNA template prepared from c724/UAS::GC-A tubules. Lane 3: cDNA template prepared from c710/UAS::GC-A tubules. Lane 5: cDNA template prepared from c42/UAS::GC-A tubules, using intron-flanking PKC primers (appendix 1) as a control for genomic contamination. Lane 6: PCR using wild-type genomic DNA as a negative control.

3.3.3 Western analysis of GC-A transgenic flies.

Using Western blot analysis, the expression levels of GC-A protein in heat-shocked and non heat-shocked hs::GC-A flies were examined. Protein samples from heat-shocked and non heat-shocked hs::GC-A flies were analysed against protein from Ore R flies using an antiserum against the last eight amino acids of GC-A (Glu-Arg-Gly-Cys-Ser-Th-Arg-Gly), raised in rabbit (R1214, kind gift of David Garbers, University of Texas SouthWestern Medical Centre at Dallas). Detection of a 180 kDa band in protein samples from heat-shocked hs::GC-A flies, that was not detectable in other samples, confirmed the presence of GC-A in these flies (Figure 3.4).

3.3.4 Immunocytochemical localisation of GC-A.

Using the GC-A specific antiserum (R1214) the cellular distribution of GC-A protein was examined in tubules from hs::GC-A flies, and from the progeny of crosses between UAS::GC-A flies and GAL4 enhancer trap lines, in which expression of GC-A could be expected to be targetted to specific cell types. Results obtained are shown in Figure 3.5 and show that GC-A was localised to the basolateral membrane of tubules (Figure 3.5B). In tubules from progeny of crosses between UAS::GC-A flies and c42 flies, localisation was shown to be confined to the principal cells of the main segment of tubules, with no apparent localisation to stellate cells (Figure 3.5A & B). Similarly, in tubules from progeny of a cross between UAS::GC-A flies and c724 flies, localisation of GC-A is confined to stellate cells, with the exclusion of principal cells (Figure 3.5C & D). These patterns of GC-A expression are consistent with patterns of GAL4 expression.

Tubules from heat-shocked hs::GC-A flies showed ubiquitous expression of GC-A and basolateral targetting of GC-A (Figure 3.6A & B).

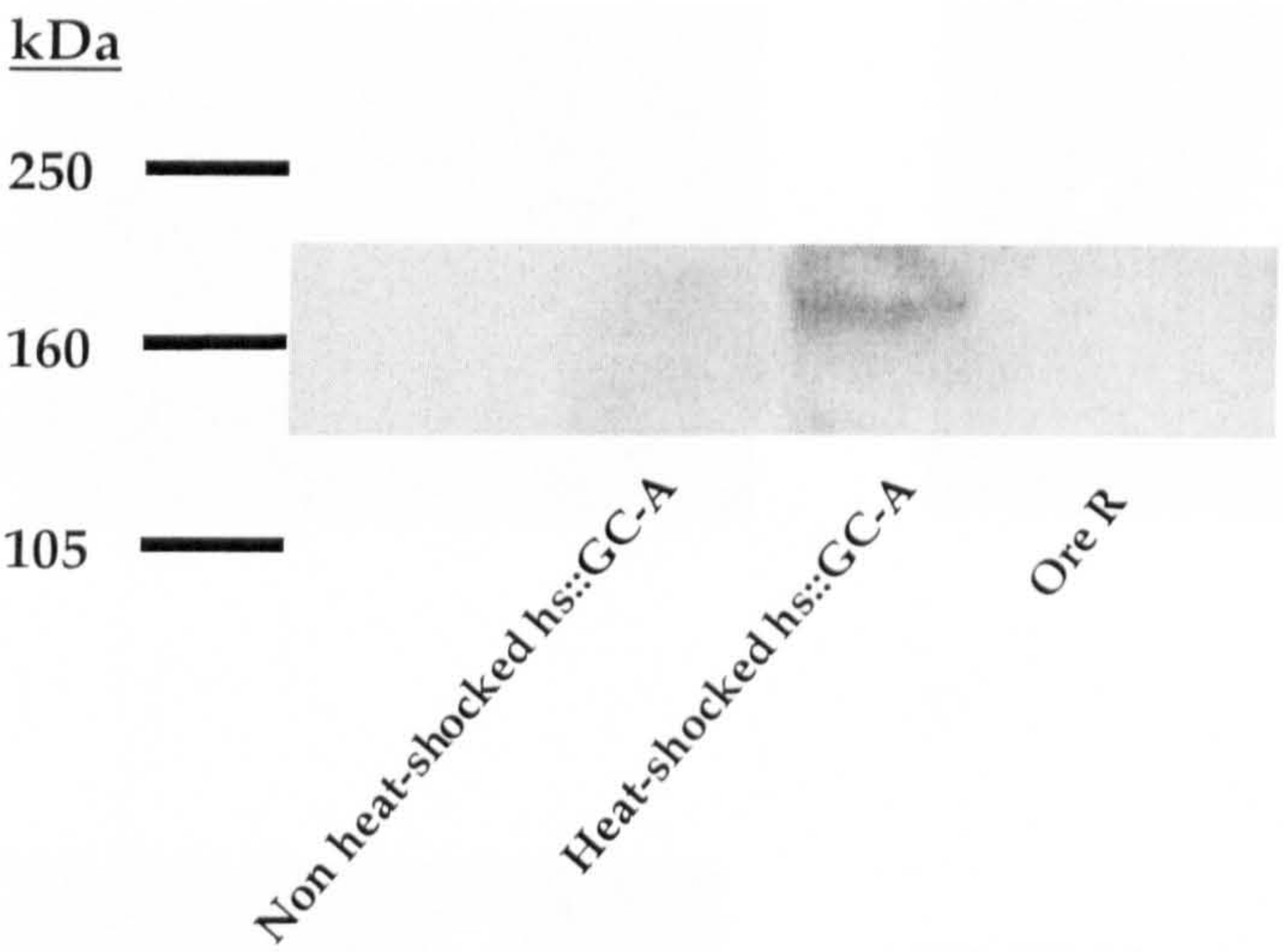

Figure 3.4: Western analysis with GC-A specific antibody

Protein samples were prepared from whole flies as described in Materials and Methods. Samples were run on a 4-15% Tris-acetate gel. Western blot analysis was carried out as described in Materials and Methods, with a 1:7000 dilution of R1214 rabbit GC-A antiserum (kind gift, David Garbers). Anti-rabbit second antibody was also used at a dilution of 1:7000. Protein markers are indicated to the left of the figure.

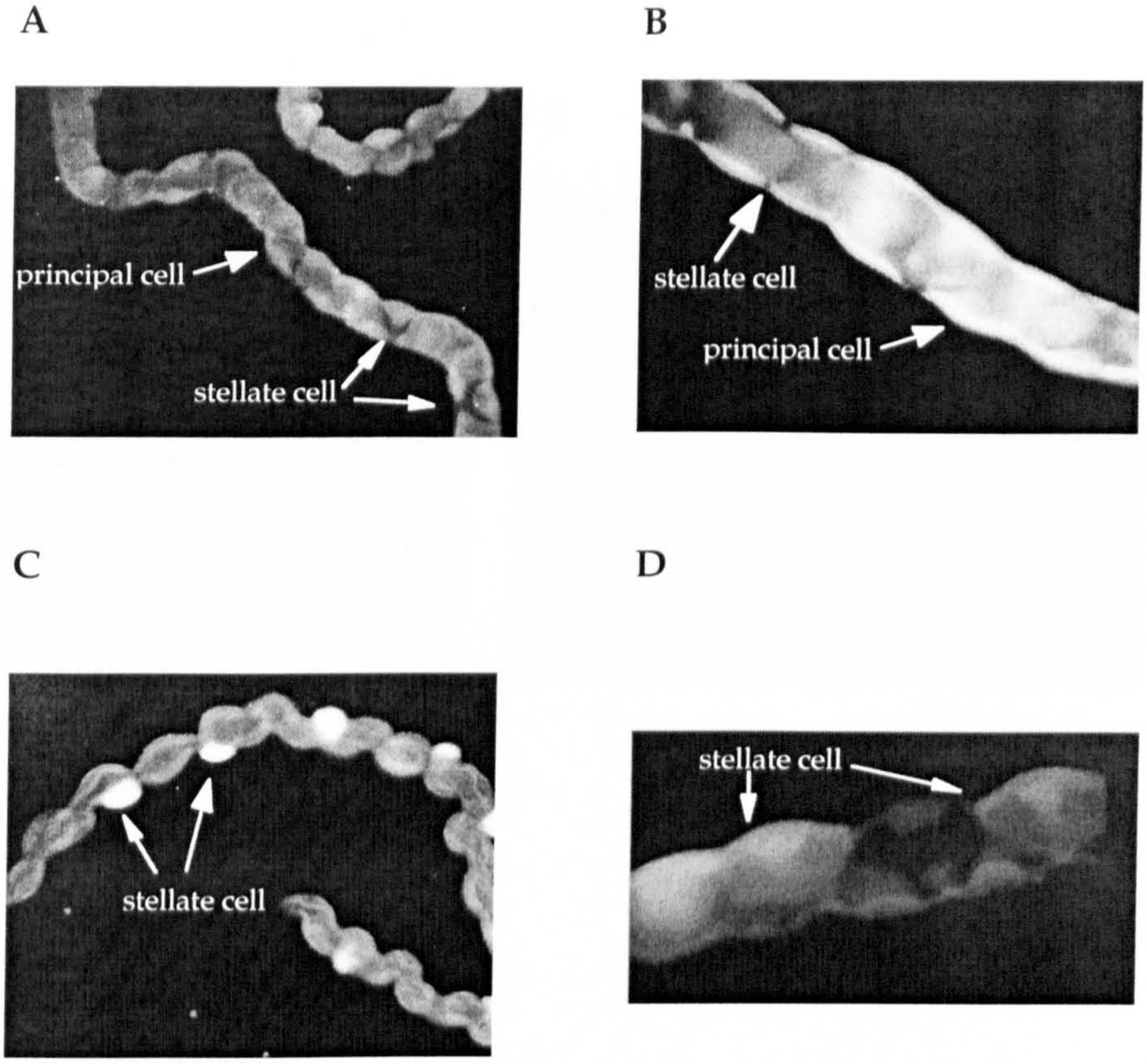

Fig 3.5: Anti-GC-A immunocytochemical analysis reveals cell-specific localisation of GC-A in transgenic tubules

Tubules were prepared as described in Materials and Methods. Tubules were incubated with rabbit R1214 anti-GC-A antiserum (kind gift, David Garbers, Howard Hughes Medical Institute, Texas) and then with fluorescein-conjugated anti-rabbit second antibody (SAPU). Tubules were viewed under UV light. A & B: GC-A is confined to principal cells in the main segment of tubules from progeny of a cross between c42 GAL4-driver line and a UAS::GC-A line. A: Expression of GC-A is excluded from stellate cells (200 x magnification). B: GC-A is targetted to the basolateral membrane of principal cells (400 x magnification). C & D: GC-A is confined to type II (stellate) cells of tubules from progeny of a cross between c724 GAL4-driver line and a UAS::GC-A line (C: 200 x magnification; D: 400 x magnification).

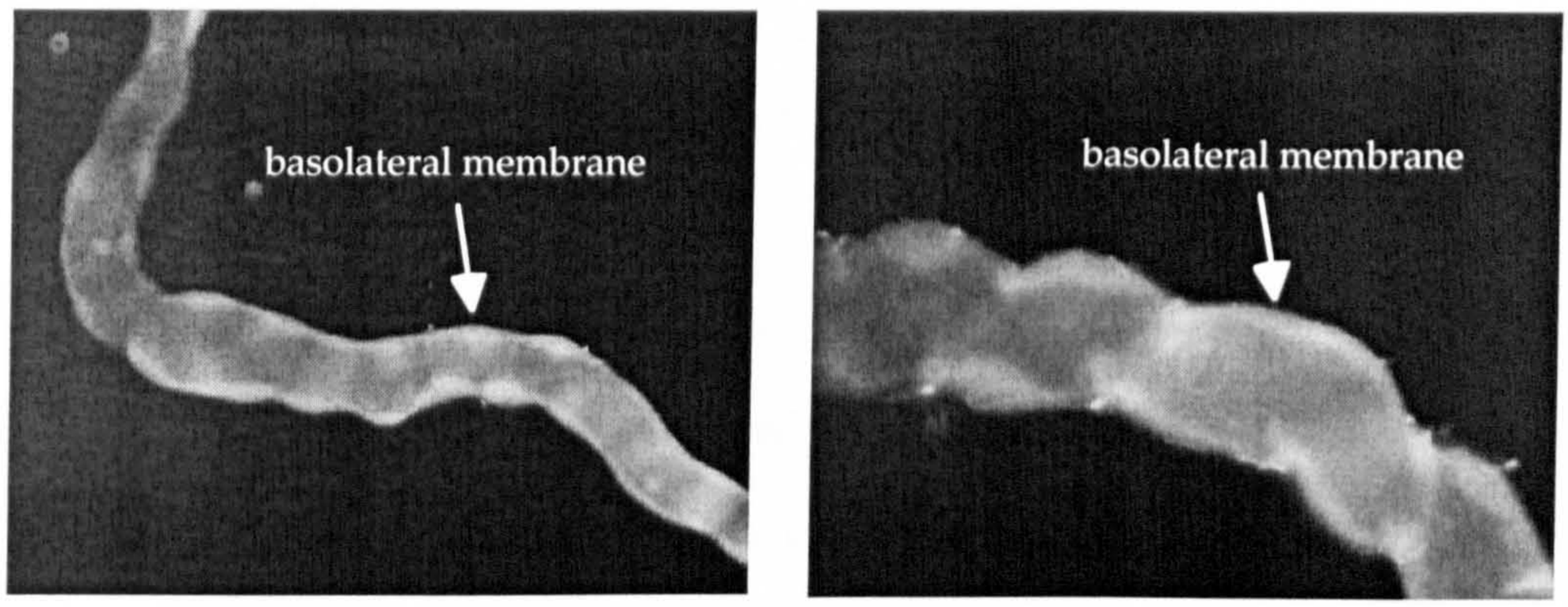

Figure 3.6: Heat-shocked hsGC::A express GC-A throughout tubules

Tubules from heat-shocked hsGC-A flies were treated with R1214 anti-GC-A antiserum (Kind gift, David Garbers, Howard Hughes Medical Institute, Texas) and fluorescein-conjugated secondary antibody.

GC-A expression was ubiquitous throughout tubules (A: 200 x magnification) and targetted to the basolateral membrane (B: 400 x magnification).

3.3.5 Conditional expression of the GC-A transgene confers tubule sensitivity to ANP.

Flies from the strain pP{CaSpeRhs::GC-A} which conditionally express GC-A under heat-shock (referred to as hs::GC-A) were heat-shocked at 37°C for 45 min 12-18 h before use. Tubules from these flies were then dissected for both fluid secretion assays and cGMP assays. These flies displayed sensitivity to ANP, the ligand for GC-A. Fluid secretion rate was measured for 30 min before ANP was added, over a range of concentrations, to the bathing solution. The fluid secretion rate was measured for a further 30 min. Figure 3.7 shows the results of fluid secretion assays on heat-shocked hs::GC-A flies. Significant changes from basal fluid secretion rates were observed in tubules treated with $\geq 10^{-10}$ M ANP, with an estimated mean EC_{50} of 7.2×10^{-10} M. Due to variation in the basal fluid secretion rates of Malpighian tubules, the percentage rise in fluid secretion rate compared to basal was taken as the most fitting and consistent measurement of the efficacy of ANP. This value was calculated as shown below:

$$\text{Percent change} = \frac{(\text{max. stimulated rate} - \text{average basal rate})}{\text{average basal rate}} \times 100$$

where max. stimulated rate is the maximum fluid secretion rate recorded post-ANP stimulation, and average basal rate is the mean of three measurements taken prior to ANP stimulation.

Figure 3.8 shows typical fluid secretion assays on tubules from flies expressing GC-A principal cell-specifically (Figure 3.8A) or stellate cell-specifically (Figure 3.8B), with the addition of ANP at 30 min.

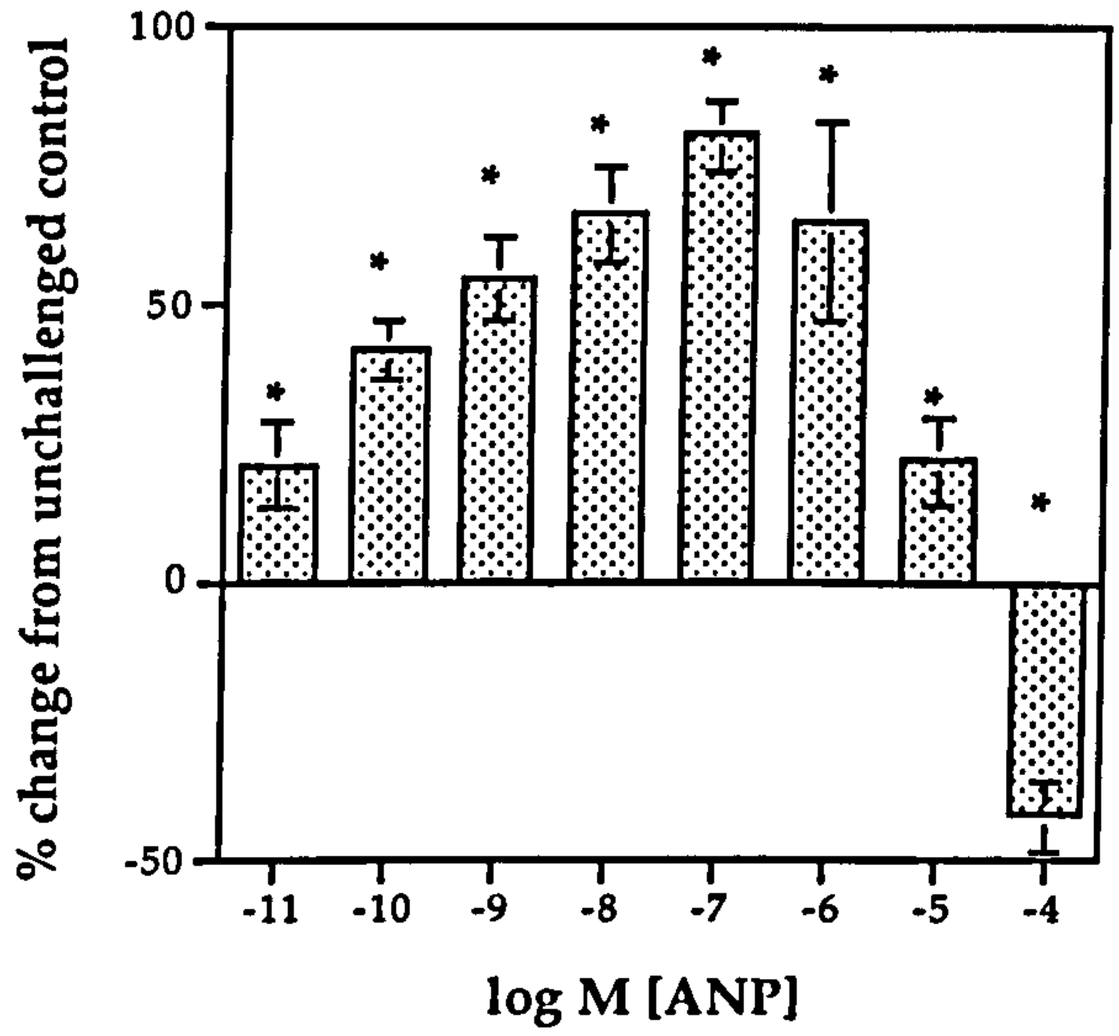

Figure 3.7: Fluid secretion is elevated in hs::GC-A tubules by ANP in a dose-dependent manner

Tubules from heat-shocked hs::GC-A were challenged with ANP at 30 min.

Tubules challenged with ANP showed elevated fluid secretion rates over basal controls at physiological levels of ANP. At high ANP concentration (10^{-4}M), fluid secretion was inhibited. Results are expressed as a percentage change from basal secretion rates ± SEM, $n \geq 10$.

Secretion rates significantly different from basal are denoted by *. ($P<0.05$, determined with the Student's *t*-test on unpaired samples, assuming unequal variances).

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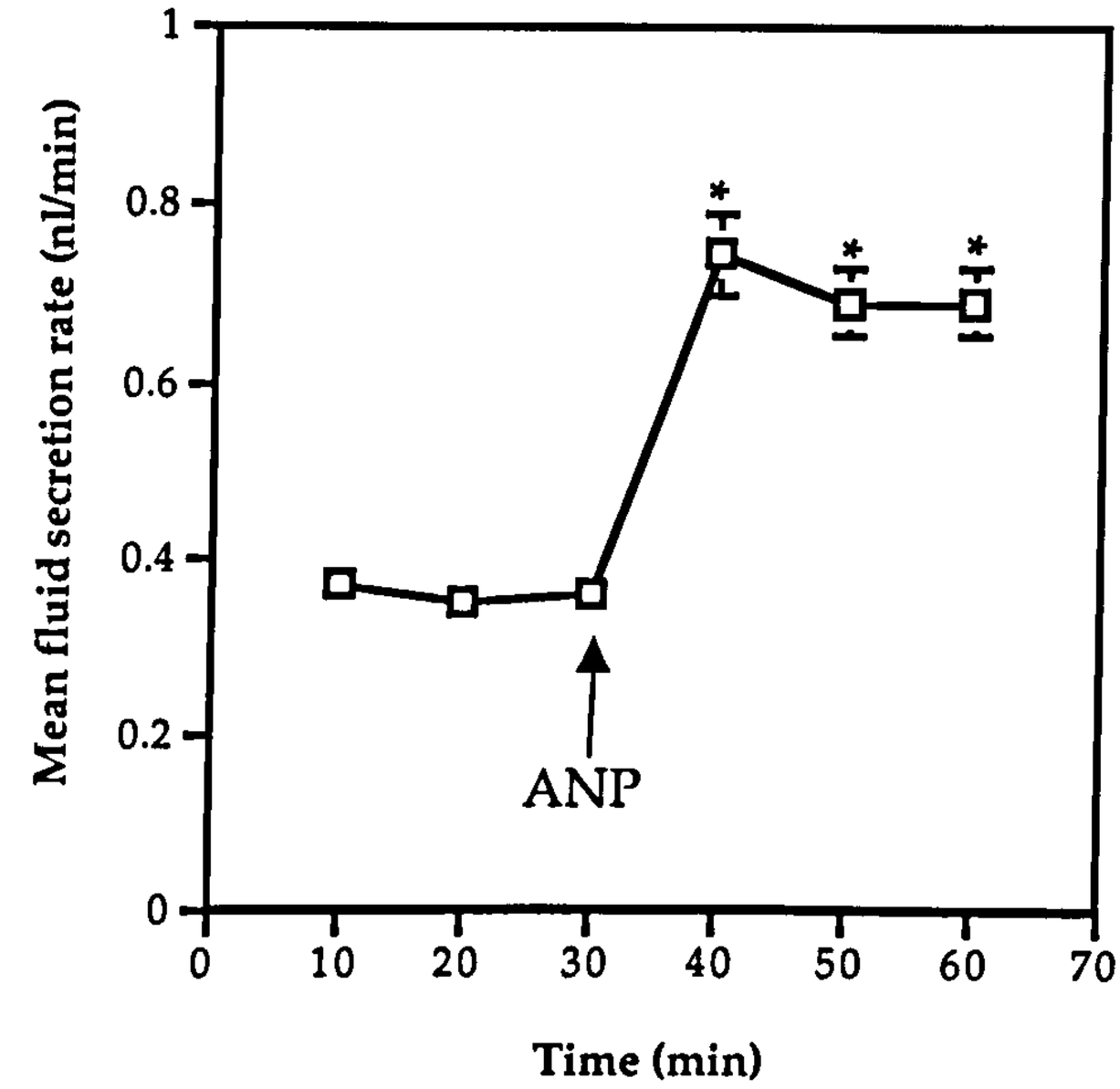

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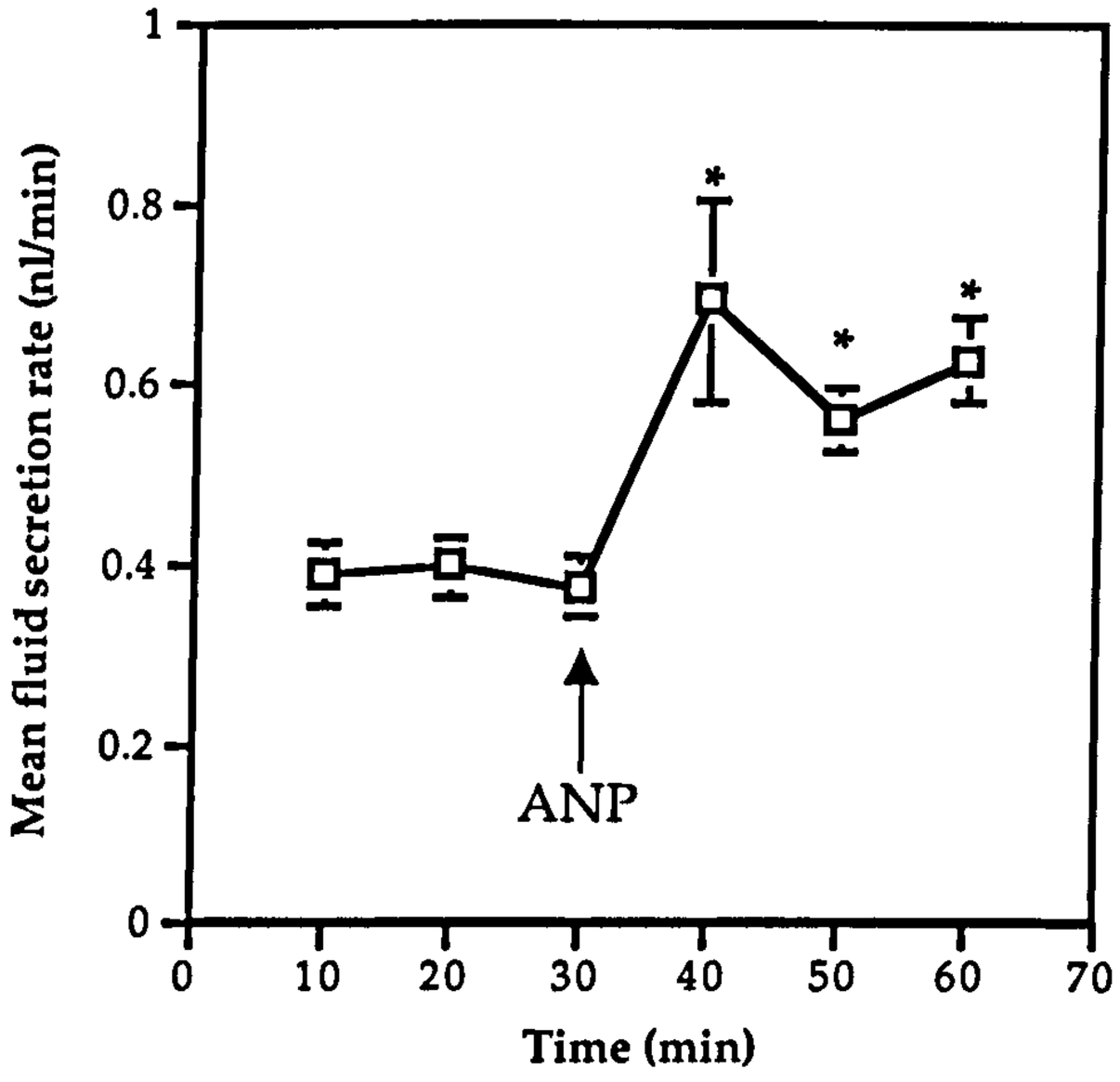

Figure 3.8: Targetted expression of GC-A transgene confers ANP-stimulated elevation of fluid transport

Basal secreton rates were measured for 30 min before tubules were treated with 1 μM ANP (arrow). Secretion rates were measured for a further 30 min.

A: Tubules from c42/UAS::GC-A flies. Results shown are mean fluid secretion rate (nl/min) Å SEM, n=10.

B: Tubules from c710/UAS::GC-A flies. Results shown are mean fluid secretion rate (nl/min) Å SEM, n=10.

Secretion rates significantly different from basal are denoted *. ($P<0.05$, determined with Student's *t*-test on unpaired samples, assuming unequal variances).

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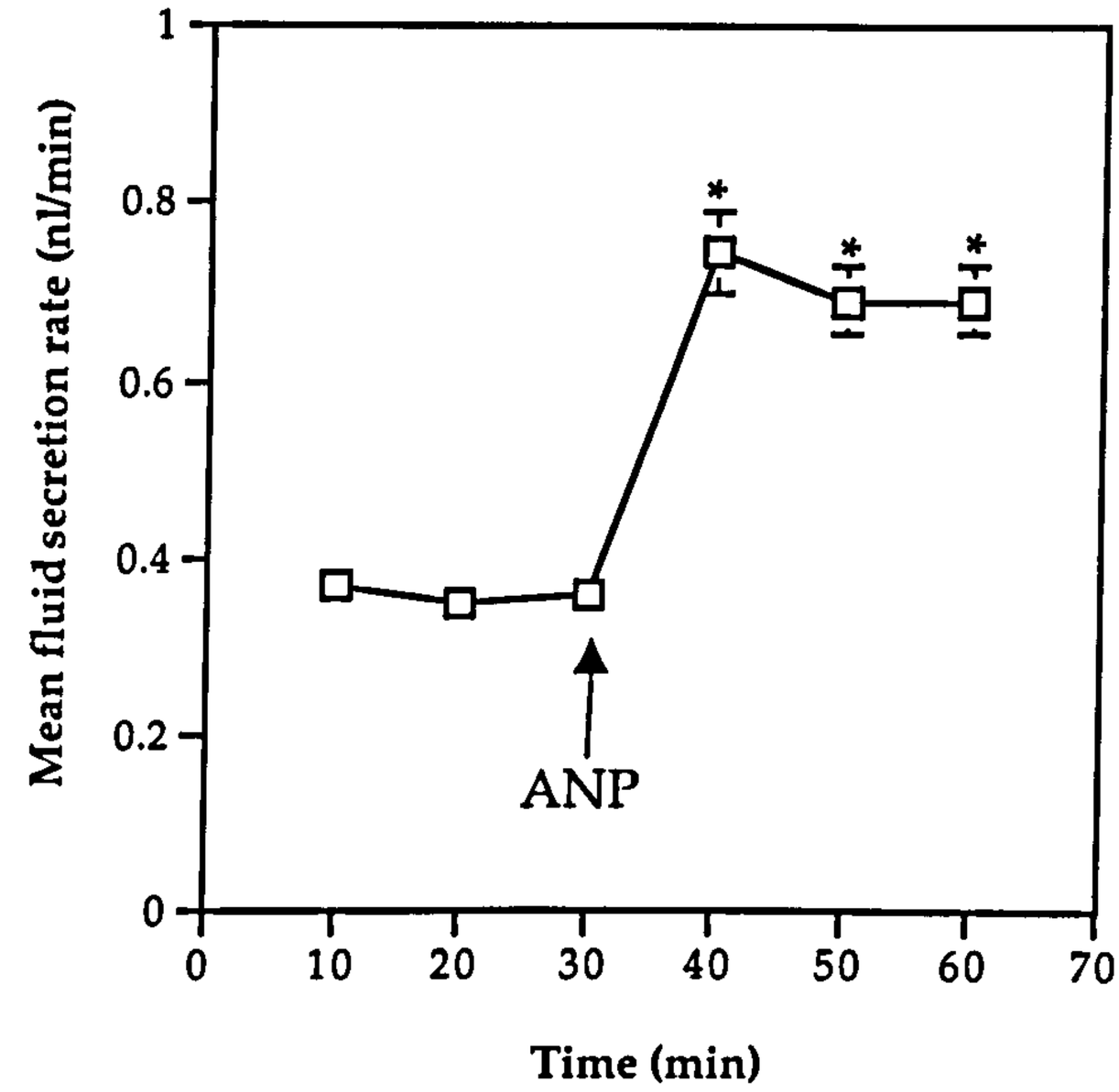

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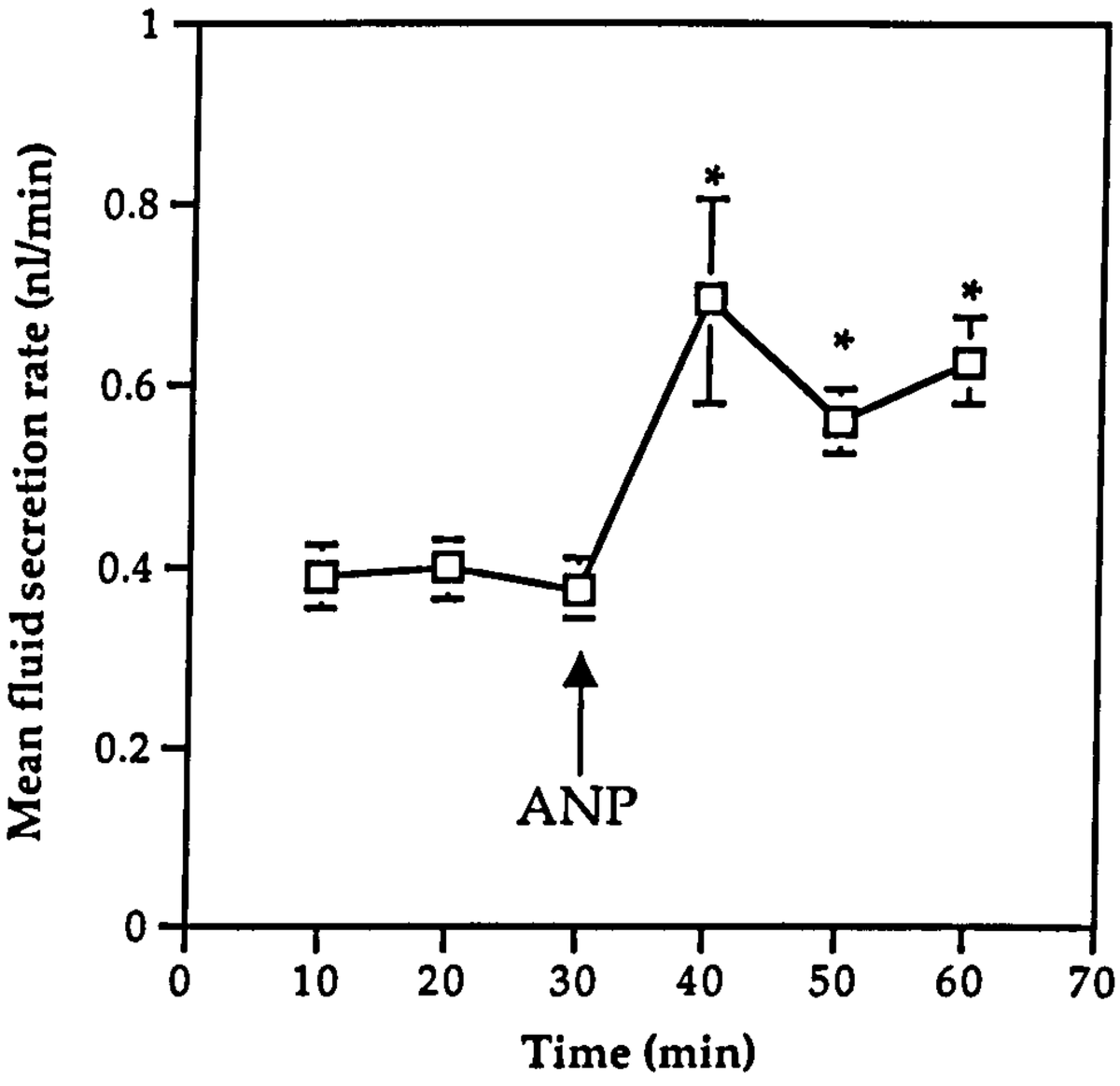


Figure 3.8: Targetted expression of GC-A transgene confers ANP-stimulated elevation of fluid transport

Basal secreton rates were measured for 30 min before tubules were treated with 1 μM ANP (arrow). Secretion rates were measured for a further 30 min.

A: Tubules from c42/UAS::GC-A flies. Results shown are mean fluid secretion rate (nl/min) ± SEM, n=10.

B: Tubules from c710/UAS::GC-A flies. Results shown are mean fluid secretion rate (nl/min) ± SEM, n=10.

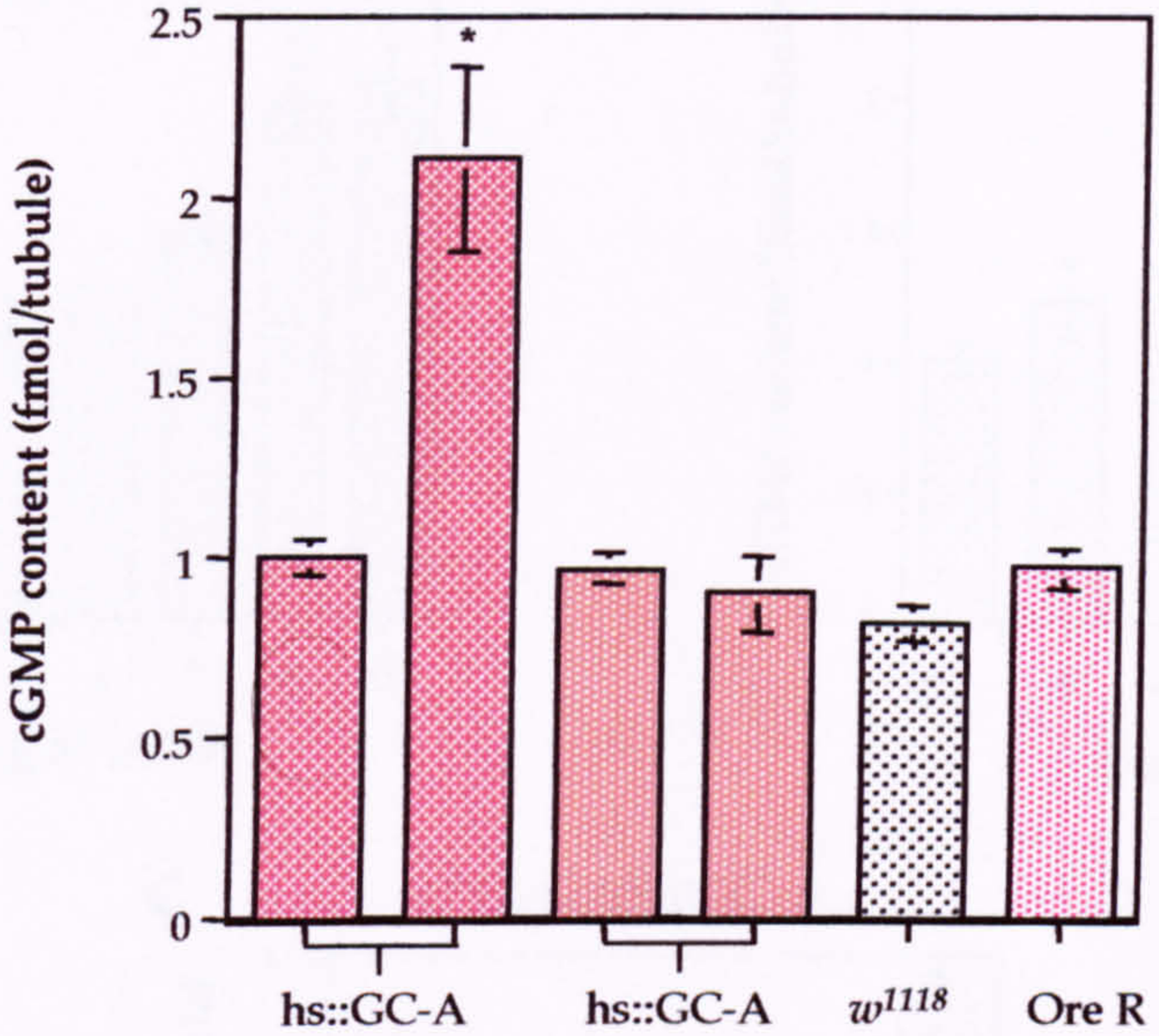
Secretion rates significantly different from basal are denoted *. ($P<0.05$, determined with Student's *t*-test on unpaired samples, assuming unequal variances).

3.3.6 ANP-stimulated tubules expressing the GC-A transgene elevate intracellular cGMP levels.

Cyclic GMP levels were assayed in tubule samples from heat-shocked hs::GC-A flies in either the absence or the presence of 0.1 μM ANP. These samples were compared to cGMP levels in tubule samples from non heat-shocked hs::GC-A flies in either the absence or the presence of 0.1 μM ANP and control samples from heat-shocked wild type flies treated with 0.1 μM ANP (Figure 3.9). All samples showed similar intracellular levels of cGMP (approximately 1 fmol/tubule), with the exception of the heat-shocked hs::GC-A sample which had been treated with ANP. This sample contained approximately double the cGMP content, indicating that the GC-A receptor had been stimulated to generate the second messenger.

The expression of the GC-A gene was also targetted to specific cell types in the tubule, by crossing UAS::GC-A flies with c42, c724 and c710 GAL4 enhancer trap lines (Figure 3.10). Tubules from progeny of these crosses were sensitive to ANP, as reflected by the elevation of intracelluar cGMP. Furthermore, the quantity of intracellular cGMP generated was directly proportional to the concentration of ANP used in the assay, with a mean EC_{50} for production of cGMP calculated to be 2×10^{-8} M.

Tubules with GC-A localised to principal cells gave rise to larger increases in cGMP than tubules expressing GC-A stellate cell-specifically, which reflects the greater volume and number of principal cells in tubules. Nevertheless, despite their smaller size and number (estimated to be 1-2% of total tubule volume), stellate cells were still able to produce a surprising amount of cGMP in response to ANP.



Heat-shock	+	+	-	-	+	+
ANP	-	+	-	+	+	+

Figure 3.9: ANP-stimulated cGMP elevation in heat-shocked hs::GC-A & wild-type tubules

Flies were heat-shocked at 37 for 1h, 12-18 h prior to use. Tubules were treated with 0.1 μM ANP and the cGMP-specific phosphodiesterase inhibitor Zaprinast (10^{-6} M) for 10 min, and then prepared as described in Materials and Methods. Results are expressed as mean cGMP content (fmol/tubule) ± SEM, n≥6. cGMP content significantly different from basal cGMP content is denoted by *. ($P<0.05$, determined with Student's *t*-test on unpaired samples, assuming unequal variances).

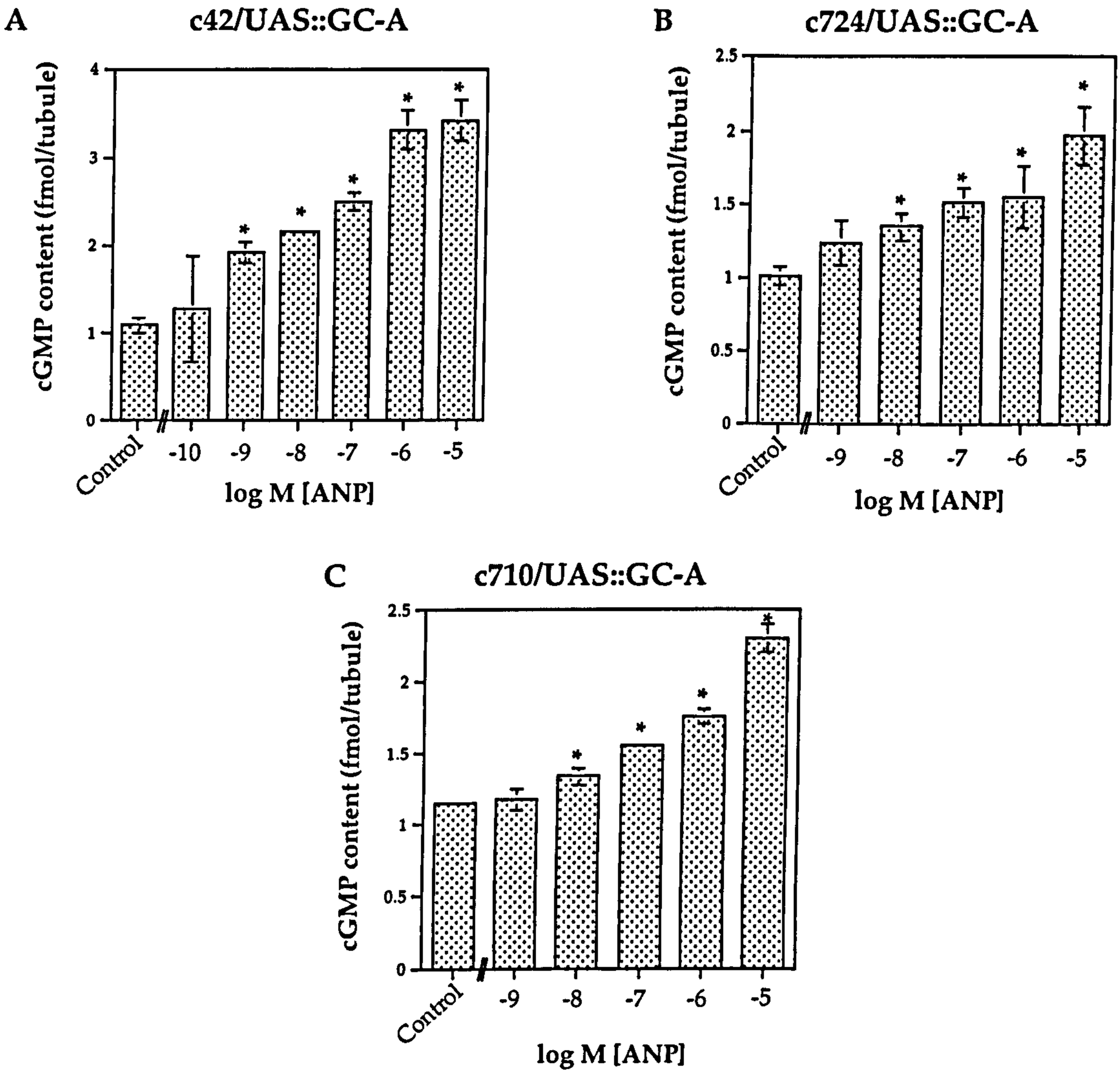


Figure 3.10: cGMP content in transgenic tubules is elevated by ANP in a dose dependent manner

Tubules were incubated with ANP and the cGMP-specific phosphodiesterase inhibitor Zaprinast (10^{-6} M) for 10 minutes. Control tubules were incubated with Zaprinast only (10^{-6} M).

A: Tubules from progeny of a cross with the principal cell-specific GAL4 driver line c42 and a UAS::GC-A line.

B: Tubules from progeny of a cross with the stellate cell-specific GAL4 driver line c724 and a UAS::GC-A line.

C: Tubules from progeny of a cross with the stellate cell-specific GAL4 driver line c710 and a UAS::GC-A line.

Results are expressed as mean cGMP content (fmol/tubule) ± SEM, n≥6. cGMP content significantly different from control sample is denoted by *. ($P<0.05$, determined with Student's *t*-test on unpaired samples, assuming unequal variances).

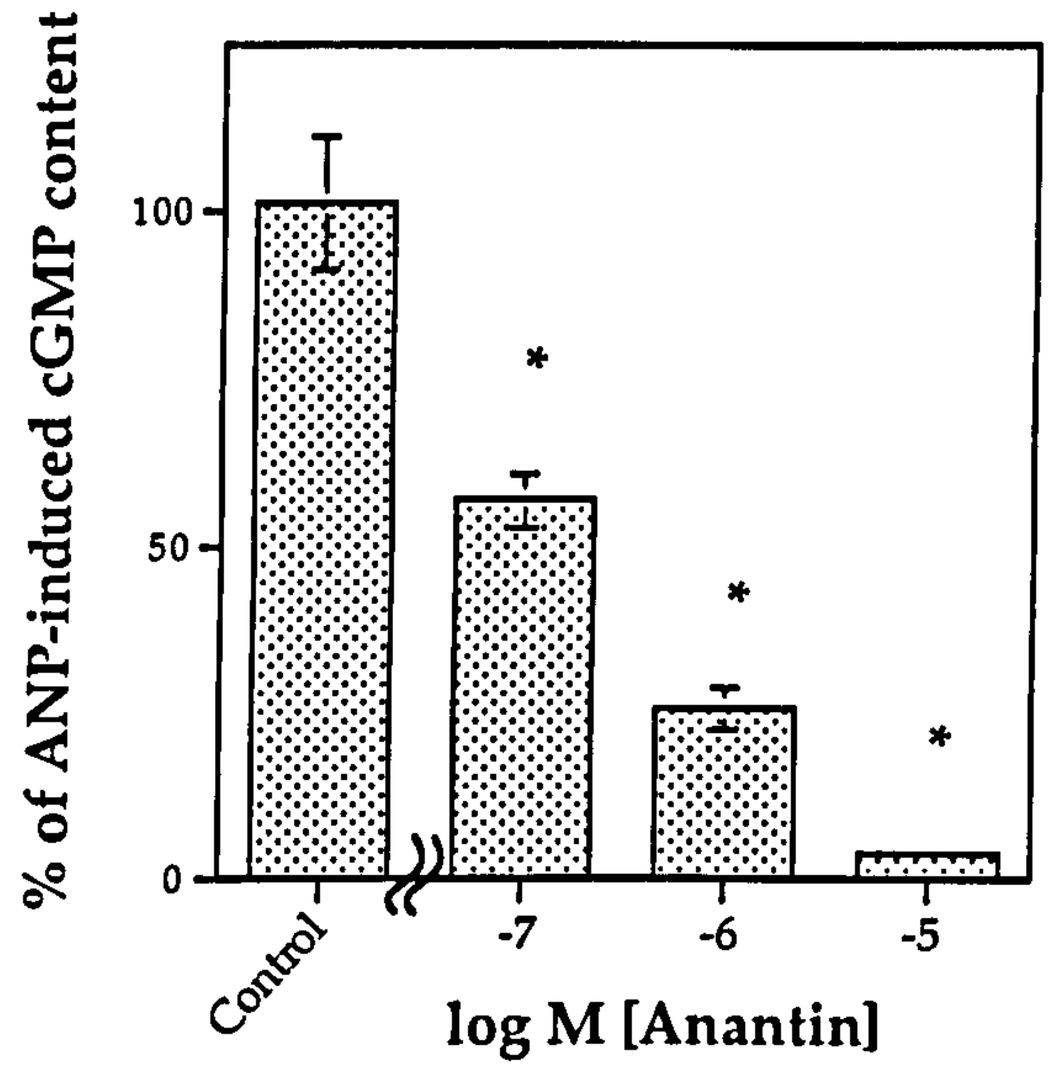
3.3.7 Anantin inhibits ANP-induced fluid secretion and cGMP production in GC-A transgenic tubules

Tubules transgenic for GC-A share a common pharmacology with COS-7 cells transfected with GC-A (Schulz *et al*, 1989). The ANP-induced effects, on both fluid secretion rate and intracellular cGMP levels in tubules, were assayed in the presence of the GC-A competitive antagonist, anantin.

The addition of the competitive inhibitor anantin to GC-A transgenic tubules was shown to attenuate the ANP-induced rise in fluid secretion (Figure 3.11A). The production of cGMP in tubules co-presented with anantin (at various concentrations) and 1 μM ANP was assayed using tubules from the progeny of a cross between UAS::GC-A flies and the principal cell specific GAL4 enhancer trap line c42. There was a dose dependent attenuation of cGMP production in these samples. On stimulation with 1 μM ANP, both production of cGMP and elevation of fluid secretion were almost completely attenuated with 10^{-5} M anantin.

The concentration of anantin needed to cause 50% inhibition of maximal cGMP production was calculated to be approximately $1x10^{-7}$ M.

A



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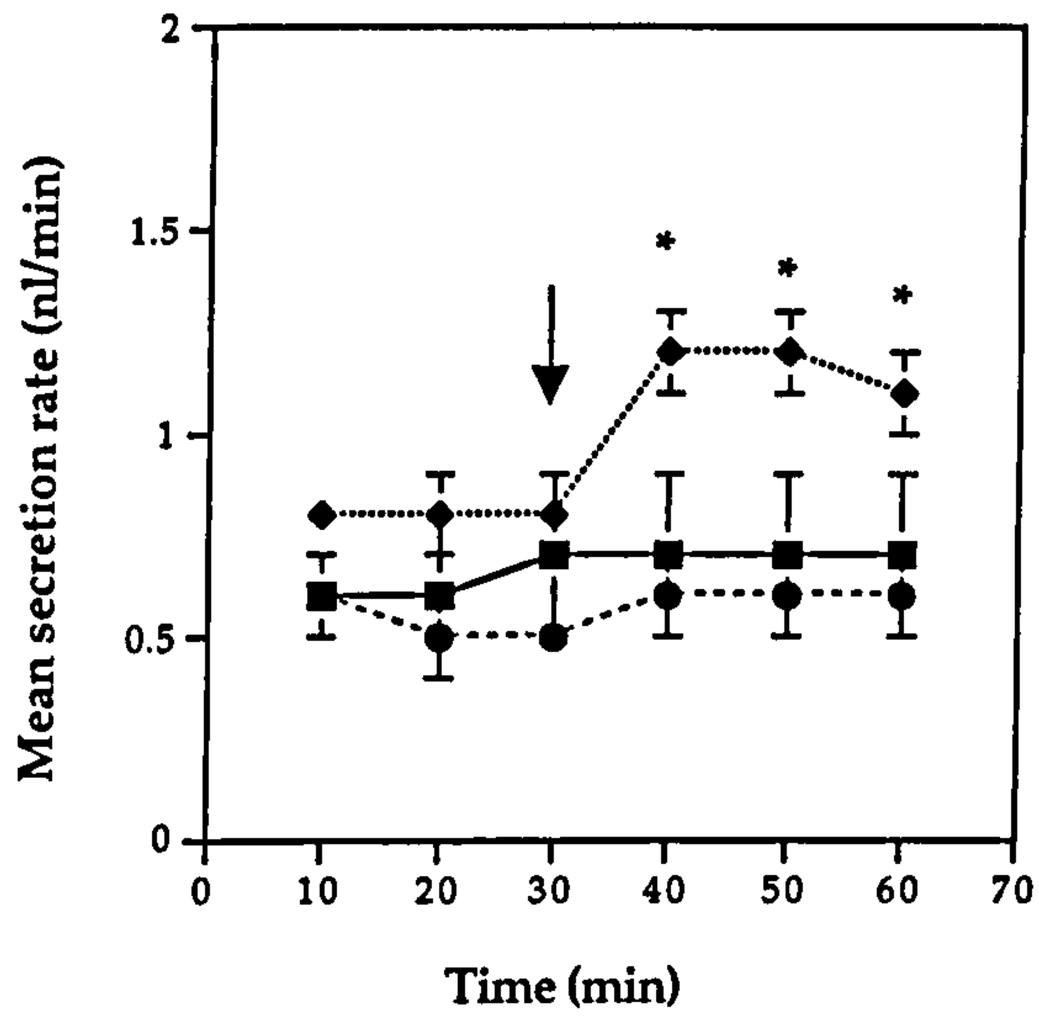


Figure 3.11: Anantin inhibits ANP-induced cGMP elevation and fluid secretion in GC-A transgenic tubules

A: Tubules from progeny of a cross with c42 (principal cell GAL 4 driver line) and UAS::GC-A were treated with 1 μM ANP, the cGMP-dependent phosphodiesterase inhibitor Zaprinast (10^{-6} M) and varying concentrations of the GC-A-specific antagonist anantin for 10 min. Tubules were then prepared as described in Materials and Methods. Results are expressed as percentages of cGMP content in tubules unchallenged with anantin ± SEM, n=3. Samples significantly different from ANP-stimulated control tubules are denoted by *. ($P<0.05$, determined with the Student's *t*-test on unpaired samples, assuming unequal variances).

B: Tubules challenged with 1 μM ANP at 30 min showed elevated fluid secretion rates (◆) over non-treated tubules (●). Tubules treated with the GC-A-specific inhibitor anantin (10^{-5} M) at t=0 showed an attenuated response to ANP (■). Results shown are mean secretion rate (nl/min) ± SEM, n=10.
Secretion rates significantly different from basal are denoted by *. ($P<0.05$, determined with the Student's *t*-test on unpaired samples, assuming unequal variances).

3.3.8 Immunocytochemical localisation of cGMP.

The localisation of cGMP generated in GC-A transgenic tubules was detected by immunocytochemistry using antiserum to 2'-O-succinyl cyclic GMP conjugated to BSA raised in rabbit (Figure 3.12). Tubules from progeny of crosses with UAS::GC-A and GAL4 lines were treated with Zaprinast (10^{-5} M) alone or with 1 μM ANP and Zaprinast (10^{-5} M) for ten min before being fixed with 4 % paraformaldehyde. Tubules treated with Zaprinast alone displayed very low background levels of fluorescence with the fluorescein-conjugated anti-rabbit secondary antibody. Tubules treated with ANP and Zaprinast showed high levels of fluorescence in a cell-specific manner, consistent with the driver for the transgene.

In tubules from progeny of a cross between UAS::GC-A and c42 GAL4 flies, cGMP appeared to be localised throughout the principal cells of the main segment (Figure 3.12A), but appeared to have strongest localisation in pools at the basolateral membrane (Figure 3.12B). This would suggest that the second messenger does not diffuse rapidly throughout the cell from its site of generation. Stellate cell-specific generation of cGMP was achieved by stimulating tubules from the progeny of a UAS::GC-A cross and GAL4 line c724 with ANP and Zaprinast (Figure 3.12C). Cyclic GMP was clearly generated exclusively in these cells; however, the stellate cells were too small to determine subcellular distribution of the second messenger.

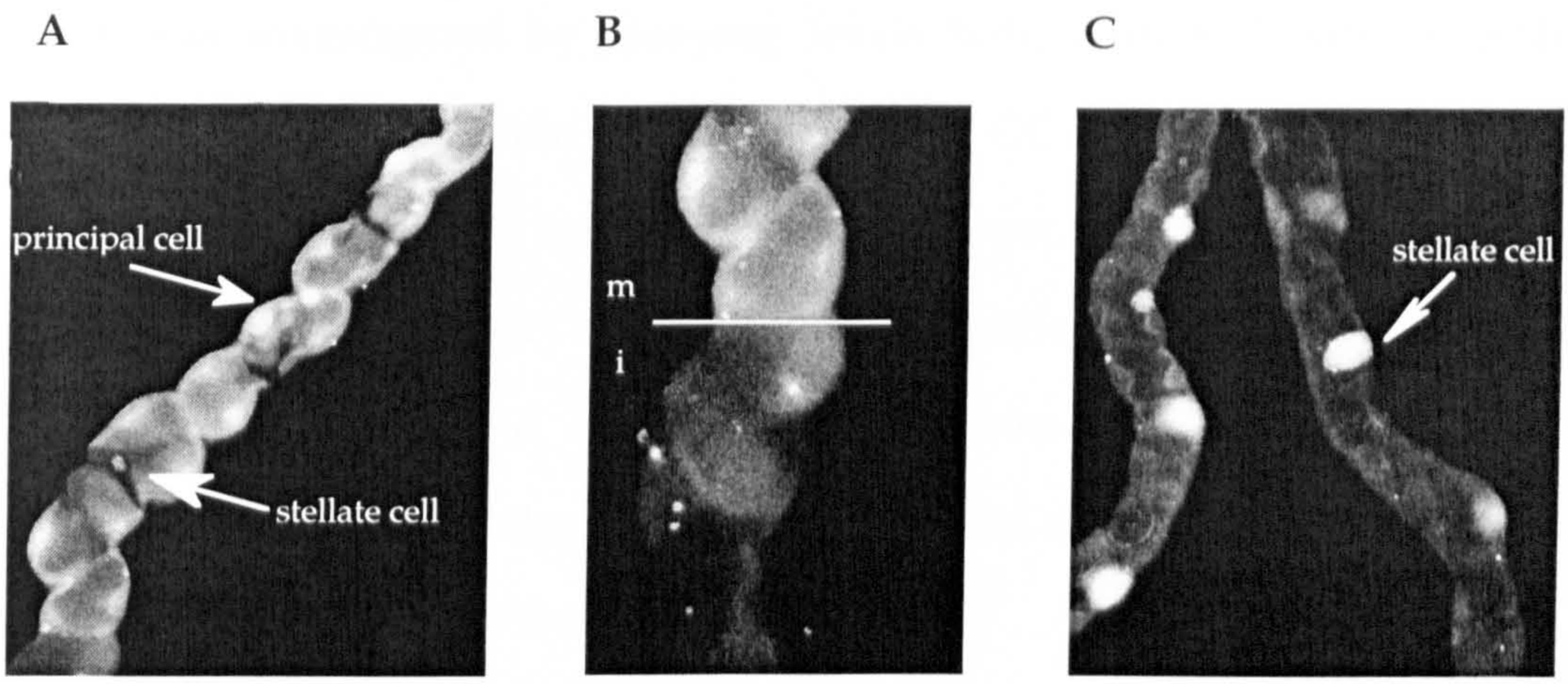


Fig 3.12: Anti-cGMP immunocytochemical analysis reveals cell-specific generation of cGMP in GC-A transgenic tubules in response to ANP

Tubules from progeny of a cross between GAL4 enhancer trap lines and UAS::GC-A lines were treated with the cGMP-specific phosphodiesterase inhibitor Zaprinast (10^{-6} M) and 1 μM ANP for ten minutes before being fixed, as described in Materials and Methods. Tubules were incubated with rabbit anti-cGMP antiserum and then with fluorescein-conjugated anti-rabbit second antibody. Tubules were viewed under UV light. A: Cyclic GMP production is confined to principal cells in the main segment of tubules from progeny of a cross between *c42* GAL4-driver line and a UAS::GC-A line (200 x magnification). B: Cyclic GMP production is not elevated in the initial segment of tubules (m=main segment; i=initial segment) (400 x magnification). C: Cyclic GMP production is confined to type II (stellate) cells of tubules from progeny of a cross between *c724* GAL4-driver line and a UAS::GC-A line (200 x magnification).

3.3.9 Cyclic AMP levels in GC-A transgenic tubules

The possibility that intracellular cAMP levels may be altered upon activation of GC-A was investigated by assaying levels both with and without ANP stimulation in samples from tubules expressing GC-A, and from a wild type control (Figure 3.13). All samples contained approximately the same basal levels of intracellular cAMP, and there was no significant change in these levels upon ANP-induced GC-A activation. This demonstrates that cGMP is the specific downstream effector of GC-A, and that its effects on cell function do not include modulation of the basal levels of cAMP.

3.3.10 $[Ca^{2+}]_i$ in ANP-stimulated GC-A transgenic tubules

In *Drosophila*, $[Ca^{2+}]_i$ is conveniently measured using a cell-specific transgenic aequorin system. GAL4 drivers are used to direct expression, as described elsewhere in this chapter. Flies homozygous for the GAL4 driver and for UAS::aequorin were crossed with flies homozygous for UAS::GC-A, and progeny heterozygous for each transgene were then used to measure $[Ca^{2+}]_i$. Intracellular Ca^{2+} levels were assayed in tubules from progeny of a cross between male UAS::GC-A flies and female UAS::apoaequorin;+/+;c42 GAL4 flies for principal cell-specific measurements and a cross between male UAS::GC-A flies and female UAS::apoaequorin;+/+;c710 GAL4 flies for stellate cell-specific measurements. Tubules responded to ANP with a rapid elevation of $[Ca^{2+}]_i$ in principal cells followed by a sustained secondary response (Figure 3.14A). Cross-talk is therefore taking place between cGMP or a cGMP-dependent effector and mechanisms dependent on Ca^{2+} in principal cells.

There was no change in $[Ca^{2+}]_i$ in stellate cells, suggesting that no cross-talk occurs in these cells (Figure 3.14B).

ANP did not cause a rise in $[Ca^{2+}]_i$ in control tubules, that only expressed aequorin and GAL4 (not shown).

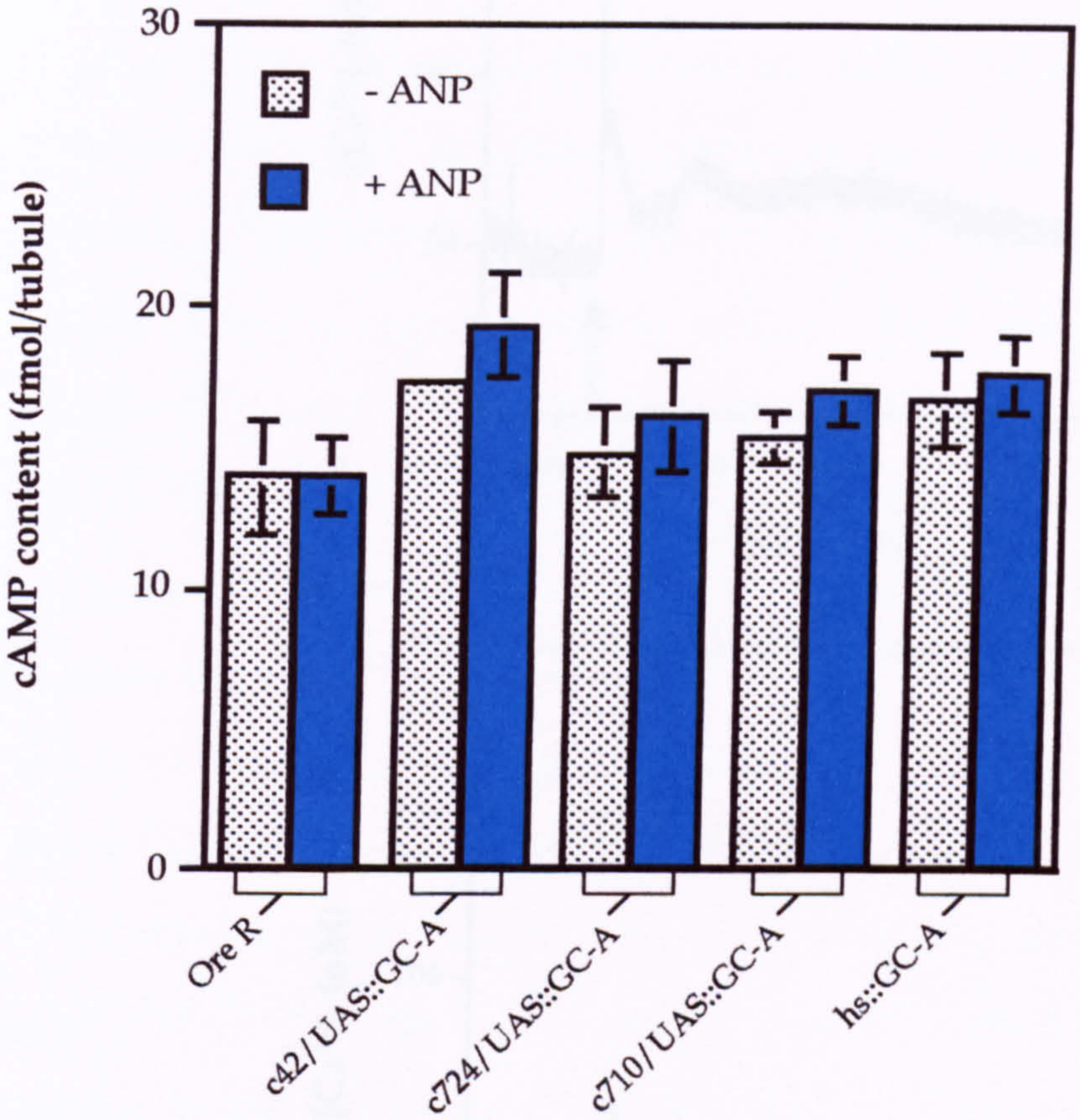


Figure 3.13: GC-A transgenic tubules do not significantly raise intracellular cAMP levels in response to ANP

Tubules were treated with ANP (1μM) and IBMX (10^{-5}M) for 10 min and prepared as described in Materials and Methods. Results are expressed as mean cAMP content (fmol/tubule) ± SEM, n=3. Differences between samples ANP-treated samples and control samples are not significant ($P>0.05$, determined with Student's *t*-test on unpaired samples, assuming unequal variances).

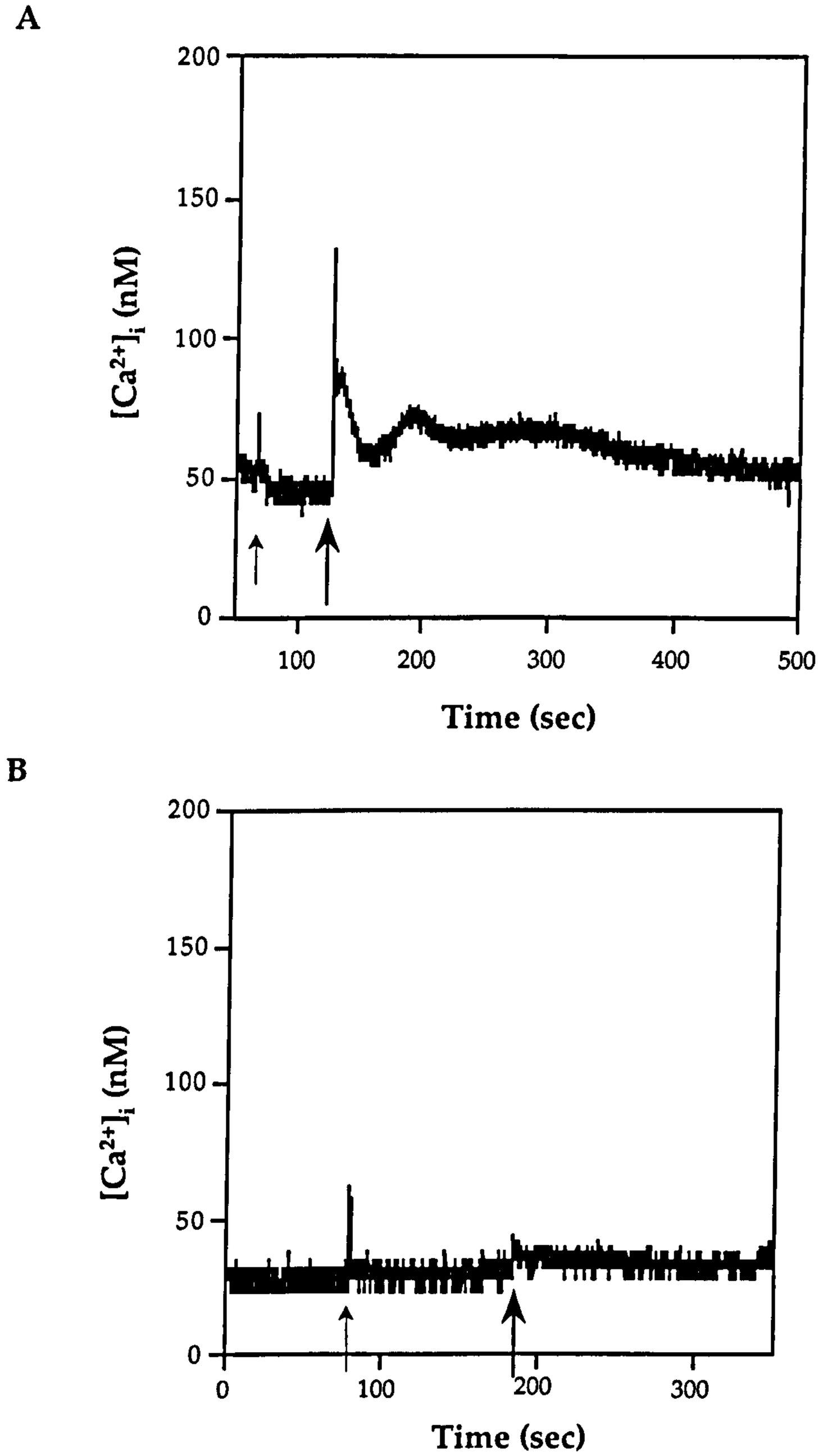


Figure 3.14: ANP-induced $[Ca^{2+}]_i$ rises in tubules expressing GC-A: representative traces

Typical traces of changes in $[Ca^{2+}]_i$ in tubule cells expressing GC-A when stimulated with 1 μM ANP (large arrows).

A: Ca^{2+} response to ANP in principal cells of aeq;+/+; c42 /GC-A tubules.

B: Ca^{2+} response to ANP in stellate cells of aeq; +/+; c710 GAL4/GC-A tubules.

The peaks prior to the main rises in $[Ca^{2+}]_i$ are control ("mock") injections of Schneider's medium (small arrows).

3.3.11 Cell-specific cyclic GMP-dependent kinase activity in GC-A transgenic tubules.

Cyclic GMP-dependent kinase activity was assayed in tubules from progeny of crosses between UAS::GC-A flies and GAL4 enhancer trap lines c42 (principal cell-specific) and c724 (stellate cell-specific) in the presence and absence of ANP. In order to determine ANP-induced changes in kinase activity, as opposed to absolute kinase activity, tubule samples were prepared in assay buffer lacking cGMP (Figure 3.15A). This ensured that kinase activity measured was directly due to endogenous intracellular cGMP generated by GC-A. Tubules were treated with the cGMP-specific phosphodiesterase inhibitor Zaprinast and stimulated with 1 μM ANP for 10 min in Schneider's medium before being transferred to assay buffer. Tubules expressing GC-A in principal cells showed nearly a 20 fold elevation above basal kinase activity when challenged with ANP, and tubules expressing GC-A in stellate cells showed a doubling in kinase activity above basal when challenged with ANP.

As a positive control for kinase activity, the assay was also conducted on samples that were treated as above, but were transferred to assay buffer containing 10 μM cGMP (Figure 3.15B). These samples were intended to measure absolute kinase activity. This assay indicated that tubules pre-treated with ANP, and therefore having generated elevated cGMP levels before being assayed, had greater kinase activity than the maximum achievable rate in samples untreated with ANP.

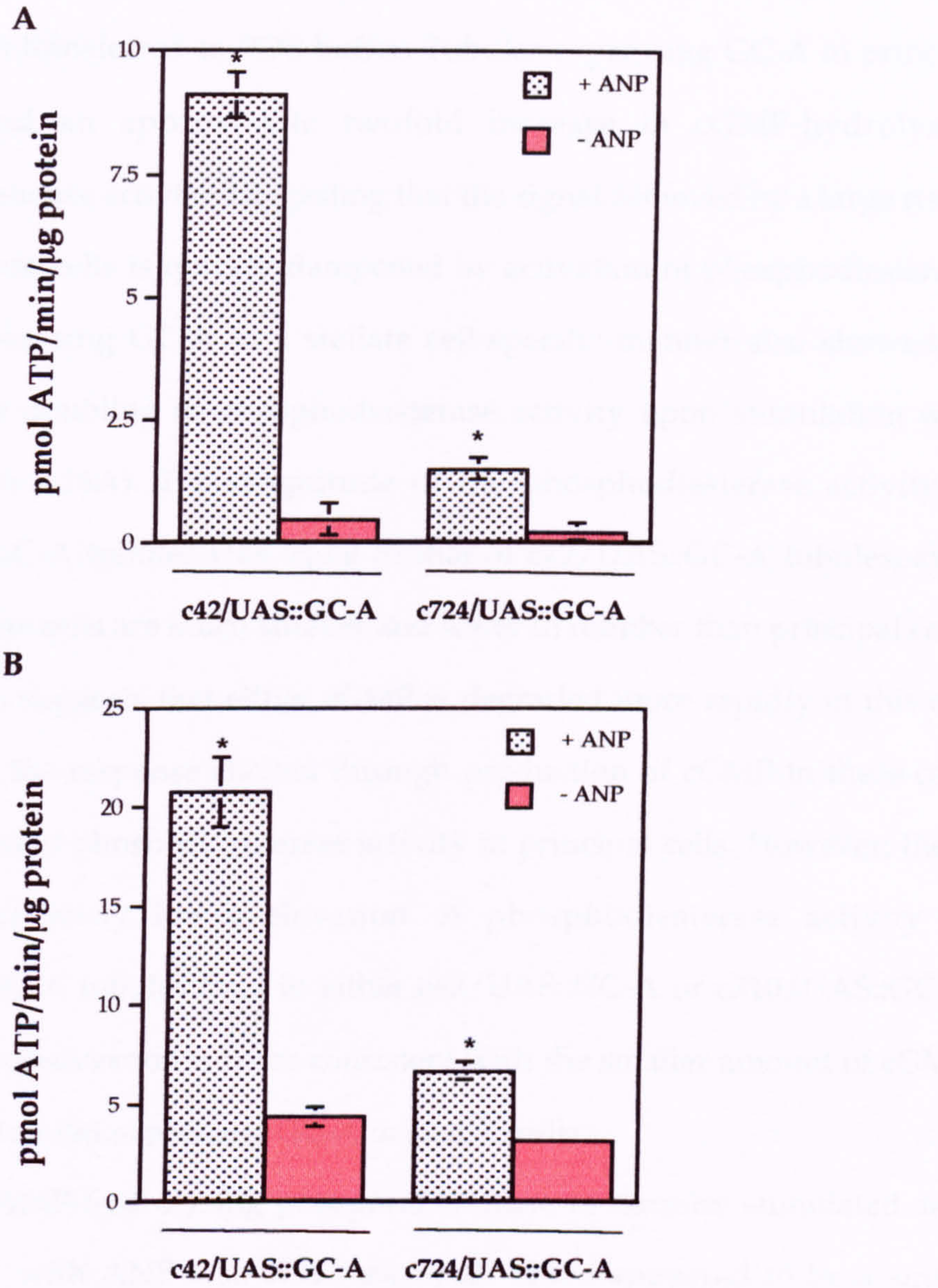


Figure 3.15: Cyclic GMP-dependent kinase activity is elevated by ANP in GC-A transgenic flies

A: Tubules from progeny of crosses between GAL4 driver lines and UAS::GC-A lines were treated with the cGMP-dependent phosphodiesterase inhibitor Zaprinast (10^{-6} M) +/- 1 µM ANP for 10 minutes. Tubules were then transferred to assay buffer and prepared as described in Materials and Methods. Results are expressed as mean kinase activity (pmol ATP/min/µg protein) ± SEM, n=6.

B: Tubules were treated as above, then transferred to assay buffer containing 10 µM cGMP. Results are expressed as mean kinase activity (pmol ATP/min/µg protein) ± SEM, n=6.

Activity significantly greater than basal is denoted *. ($P<0.05$, determined with Student's *t*-test on unpaired samples, assuming unequal variance).

3.3.12 Cell-specific phosphodiesterase activity in GC-A transgenic flies

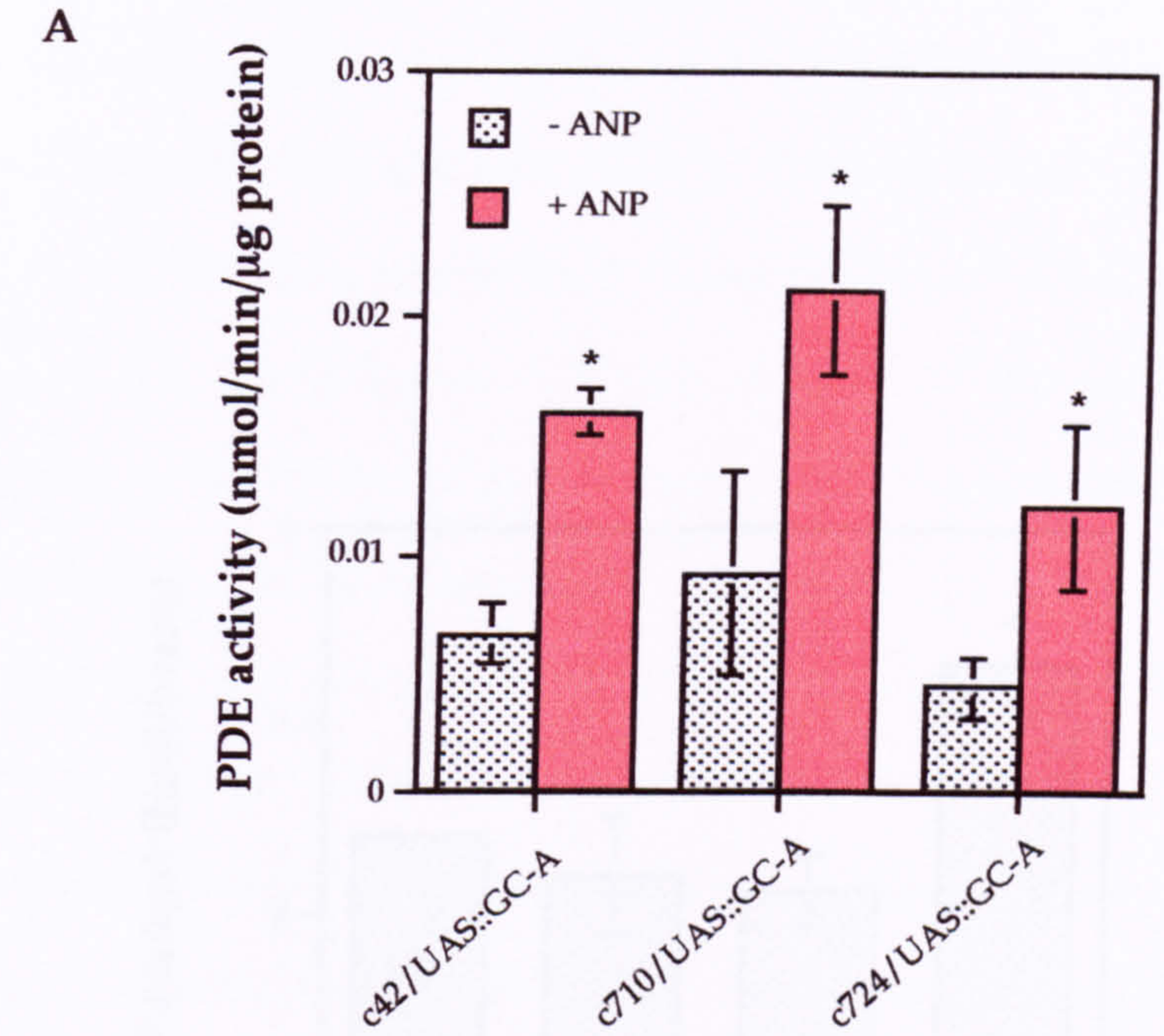
Tubules expressing GC-A in a cell-specific manner were treated with ANP for 10 min, then transferred to PDE buffer. Tubules expressing GC-A in principal cells showed an approximate twofold increase in cGMP-hydrolysing phosphodiesterase activity, suggesting that the signal achieved by a large rise in cGMP in these cells is quickly dampened by activation of phosphodiesterase. Tubules expressing GC-A in a stellate cell-specific manner also showed an approximate doubling in phosphodiesterase activity upon stimulation with ANP (Figure 3.16A). The magnitude of the phosphodiesterase activity in c710/UAS::GC-A tubules was equal to that of c42/UAS::GC-A tubules, even though stellate cells are much smaller and fewer in number than principal cells. This perhaps suggests that either cGMP is degraded more rapidly in this cell type, or that the response elicited through production of cGMP in these cells evokes increased phosphodiesterase activity in principal cells. However, there was a significantly lower elevation of phosphodiesterase activity in c724/UAS::GC-A tubules than in either c42/UAS::GC-A or c710/UAS::GC-A tubules. This observation is more consistent with the smaller amount of cGMP generated in tubules expressing GC-A in stellate cells.

Activity of cAMP-hydrolysing phosphodiesterase in samples stimulated and unstimulated with ANP was also measured. There appeared to be a small decrease in activity below basal levels in ANP-stimulated samples, which indicates that there was a cAMP-independent mechanism controlling this down-regulation, as cAMP levels were shown not to be significantly raised upon GC-A activation (Figure 3.13). This suggests that there is an element of cross talk between cGMP-hydrolysing and cAMP-hydrolysing phosphodiesterases, or that a phosphodiesterase that hydrolyses cAMP has been inhibited by cGMP. However, as with the cGMP-hydrolysing phosphodiesterase assay, there is an inconsistency between samples expressing

GC-A in stellate cells (c710/UAS::GC-A and c724/UAS::GC-A). Both samples have approximately the same basal level of activity, but only c724/UAS::GC-A samples demonstrated significant inhibition of activity.

3.3.13 GC-A as a generic tool for study of tissue-specific cGMP signalling

Flies with a P-element insertion containing GC-A can potentially be used for studying cGMP signalling in any tissue or cell type throughout the organism. This can be achieved either by heat-shocking hs::GC-A flies, resulting in ubiquitous expression of the gene, or by crossing UAS::GC-A flies with any of the huge range of GAL4 lines that are available for targetted expression: introduction of ANP to the fly, or a particular tissue, will stimulate the receptor to elevate intracellular cGMP levels. This obviously creates great scope for studying cGMP in *Drosophila*, and even behavioural phenotypes could be studied if ingestion of ANP was achieved. Figure 3.17 illustrates that this method of study can be used generically. Brains (including optic lobes) were assayed for cGMP content with and without 1 μM ANP, as in other assays, from heat-shocked and non heat-shocked hs::GC-A flies. Elevated cGMP content was only observed in brains from flies that had been treated with both heat-shock and ANP. This demonstrates that rat ANP does not normally elicit a response in this tissue, and therefore confirms the potential for GC-A to be used as a specific modulator of cGMP in tissues other than Malpighian tubules.



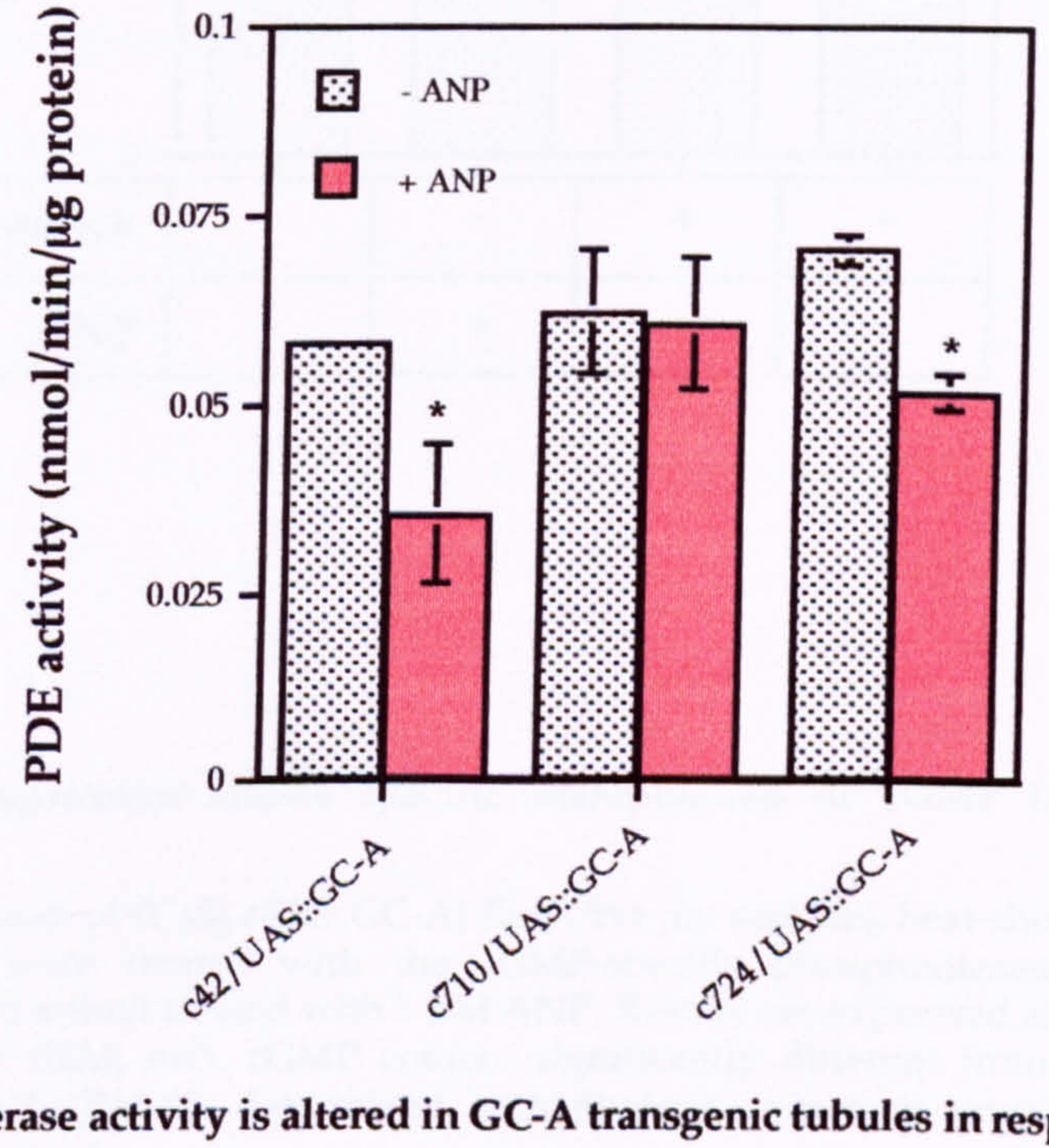


Fig 3.16: Phosphodiesterase activity is altered in GC-A transgenic tubules in response to ANP

Tubules from the progeny of crosses with GAL4 enhancer trap lines and UAS::GC-A lines were treated with 1 µM ANP for 10 min. Phosphodiesterase activity was assayed as described in Materials and Methods.

A: Samples were assayed for cGMP-dependent phosphodiesterase activity.

B: Samples were assayed for cAMP-dependent phosphodiesterase activity.

Results are expressed as mean phosphodiesterase activity (nmol/min/µg) ± SEM, n=3.

Activity significantly different to basal is denoted *. ($P<0.05$, determined with Student's *t*-test on unpaired samples, assuming unequal variance).

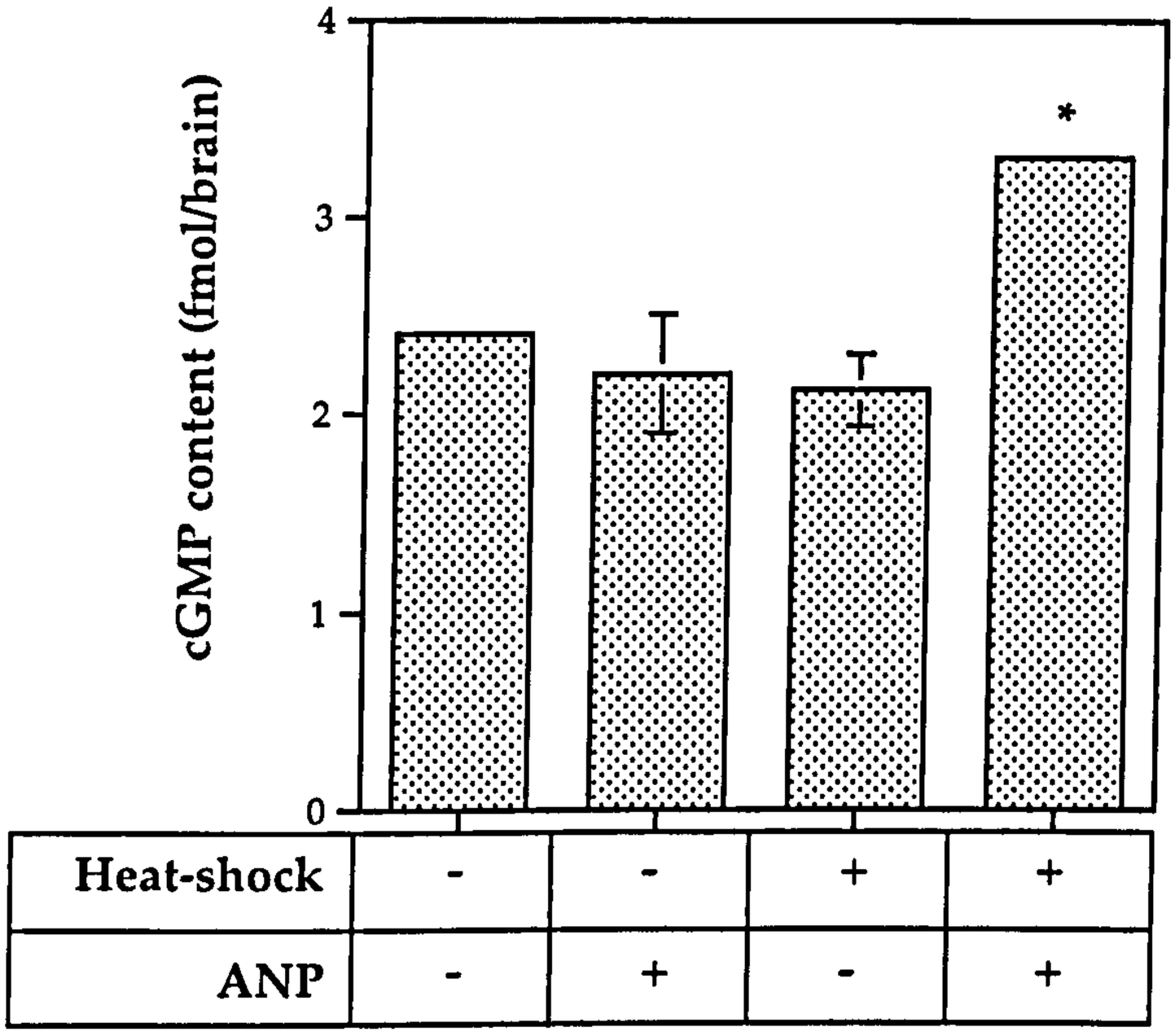


Figure 3.17: GC-A expression allows specific manipulation of cGMP in *Drosophila melanogaster* brain

Brains were dissected from pP{CaSpeRhs::GC-A} flies (five per sample), heat-shocked and non heat-shocked. Brains were treated with the cGMP-specific phosphodiesterase inhibitor Zaprinast (10^{-6} M) and a subset treated with 1 μM ANP. Results are expressed as mean cGMP content (fmol/brain) ± SEM, n=3. cGMP content significantly different from basal cGMP content is denoted by *. ($P<0.05$, determined with Student's *t*-test on unpaired samples, assuming unequal variances).

3.4 Discussion

A 3.1 kb rat GC-A open reading frame was subcloned into the P-element vectors pP{CaSpeRhs/act} and pP{UAST} to allow temporal and spatial control of expression of the gene, respectively. Transcription of GC-A was detected in tubules by reverse transcriptase PCR and a protein of approximately 180 kDa was detected in whole fly protein samples by Western analysis using an antibody raised to the last eight amino acids of GC-A. Immunocytochemical studies with the same antibody localised the protein to the basolateral membrane of tubules. Sorting of proteins to the basolateral membrane in polarised epithelia requires cytoplasmic tyrosine-based signals or cytoplasmic dileucine motifs (Brown and Breton, 2000). Therefore, GC-A might be expected to be basolaterally targetted due to the presence of a C-terminal dileucine motif. This basolateral targetting is also consistent with the fact that the ligand for GC-A, ANP, is released into the bloodstream primarily from cardiomyocytes in mammals and would therefore be bound by receptors on the outer surface of tissues without the need for internalisation.

Expression of GC-A throughout tubules conferred ANP sensitivity, and elevation of fluid secretion was achieved with low concentrations of ANP, with an EC_{50} of 7.2×10^{-10} M ($\pm 1.7 \times 10^{-10}$ M). This value for half-maximal stimulation of fluid secretion is far lower than the EC_{50} calculated for ANP-induced cGMP production (1.89×10^{-8} M ($\pm 1.052 \times 10^{-8}$ M)). However, at 0.1 mM, ANP inhibited fluid secretion, suggesting that high levels of cGMP can inhibit fluid transport. This is consistent with the inverted 'U' response to cGMP previously reported (Davies *et al*, 1995), whereby fluid transport rate is elevated by cGMP at low concentrations, but at high concentrations cGMP becomes inhibitory. At physiological ranges ANP also acted to elevate fluid secretion rate when GC-A was expressed in a cell specific manner. Surprisingly, the maximum fluid secretion rates measured in tubules expressing GC-A in stellate cells were very

similar to those measured in tubules expressing GC-A in principal cells, despite stellate cells being smaller and fewer in number. Expression of GC-A in both principal cells and stellate cells (in hs::GC-A tubules) had an additive effect on fluid secretion rate, which implies distinct roles for cGMP in different cell types (Figure 3.18). That ubiquitous expression of GC-A results in almost double the maximal rates from stimulated c42/GC-A and c710/GC-A tubules is interesting; the lower segment of tubules is known to be involved in the process of fluid reabsorption (O'Donnell and Maddrell, 1995). Therefore, one might expect that expression of GC-A, and subsequent elevation of cGMP in response to ANP, would stimulate the lower tubule and elevate reabsorption. One explanation as to why an attenuation of the increased activity in the main segment is not observed is simply that the lower tubule is not contained within the bathing medium in these assays, and therefore GC-A expressed in this segment is not exposed to ANP.

Production of the second messenger cGMP was detected immunocytochemically in transgenic tubules that were challenged with ANP, whereas background levels were very low; in principal cells cGMP appeared in pools at the basolateral membrane. This implies that there is low diffusiblity of the cyclic nucleotide, resulting in compartmentalisation. The production of cGMP was shown by radioimmunoassay to be dependent on the concentration of ANP used, and production could be attenuated by the GC-A receptor antagonist anantin.

The specificity of cGMP as a downstream effector of GC-A activation was determined by measuring intracellular cAMP levels in ANP-stimulated tubules. No significant changes in intracellular cAMP levels between stimulated and unstimulated samples were observed, suggesting that GC-A acts to specifically generate cGMP and that there is no cross-talk that leads to changes in resting levels of cAMP.

Intracellular Ca^{2+} levels were measured in both principal cells and stellate cells, and tubules expressing GC-A in principal cells responded to ANP with a biphasic elevation of $[Ca^{2+}]_i$. This is consistent with the model for CAP_{2b}/CAPA signalling in principal cells; the elevation of cGMP is thought to induce Ca^{2+} entry into principal cells. However, tubules expressing GC-A in stellate cells did not display elevation of $[Ca^{2+}]_i$ in response to ANP. This suggests that in principal cells cross-talk between cGMP or a cGMP-dependent effector and mechanisms dependent on Ca^{2+} is taking place, whereas there is no cross-talk of this nature in stellate cells. Interestingly, it has been demonstrated that calcium channels sensitive to the calcium channel antagonists verapamil and nifedipine are necessary for cGMP-induced calcium entry to tubules (MacPherson *et al*, 2001). It was also demonstrated by RT-PCR that the *Drosophila* cyclic nucleotide gated (CNG) ion channel (*cng*) is found in Malpighian tubules and must, therefore, have a role in fluid transport. Using fluorescent calcium channel antagonists, verapamil was demonstrated to bind at high affinity to basolateral membranes of main segment principal cells, suggesting that verapamil-sensitive calcium channels are not found in stellate cells (MacPherson *et al*, 2001). This is consistent with the observation that ANP-induced cGMP rises in principal cells induce Ca^{2+} entry, but ANP-induced cGMP elevation in stellate cells does not affect $[Ca^{2+}]_i$ levels. The possibility that there are other types of ion channels gated by downstream effectors of cGMP in stellate cells, is therefore plausible. For example, the role of cyclic nucleotides in stellate cells may be to regulate phosphorylation of chloride channels. Indeed, in the T84 intestinal epithelial cell line, cGMP accumulation by heat-stable enterotoxin increases chloride conductance across the membrane by cross-activation of cAK (Forte *et al*, 1992). However, it has previously been reported that cAMP and cGMP alter the activity of the V-ATPase located in principal cells, with only a negligible effect on anion conductance (O'Donnell *et al*, 1996). This evidence

would oppose the suggestion that cyclic nucleotides may regulate chloride conductance, but it does not address stellate cell-specific intracellular generation of cyclic nucleotides; rather, it implies that stellate cells are not capable of transporting cGMP across the basolateral plasma membrane.

Here it was demonstrated in both principal and stellate cells that cGMP-dependent kinase activity was elevated in response to ANP-induced elevation of $[cGMP]_i$. This suggests the possibility of direct phosphorylation of ion channels by cGK or cross-activation of cAK (which is inhibited in the cGK assay) leading to increased ion conductance across tubules.

In most signalling systems, there is the requirement for a negative feedback loop to allow a signal to be dampened, or turned off. The only known enzymatic reaction for the degradation of cyclic nucleotide is its conversion to 5'-nucleoside by cyclic nucleotide phosphodiesterases. Therefore, activity of cGMP- and cAMP-dependent phosphodiesterases in GC-A transgenic tubules was assayed, both in the presence and absence of ANP. Elevation in activity of cGMP-hydrolysing phosphodiesterases was found, but conversely a decrease in activity of cAMP-hydrolysing phosphodiesterase was observed. This cross-talk between phosphodiesterases did not appear to significantly alter the resting levels of cAMP, and perhaps would only have impact when adenylate cyclase activity was also elevated. Finally, the use of GC-A as a generic tool for manipulating cGMP in any tissue of *Drosophila* was illustrated by assaying $[cGMP]_i$ in brains taken from hs::GC-A flies, both heat-shocked and non heat-shocked in the presence and absence of ANP. The elevation of $[cGMP]_i$ in brains demonstrates that this work could be extended to the study of signal cross-talk in any tissue or cell type with which there is an accompanying GAL4 driver. This direct method for manipulation of $[cGMP]_i$, may reveal a previously masked phenotype.

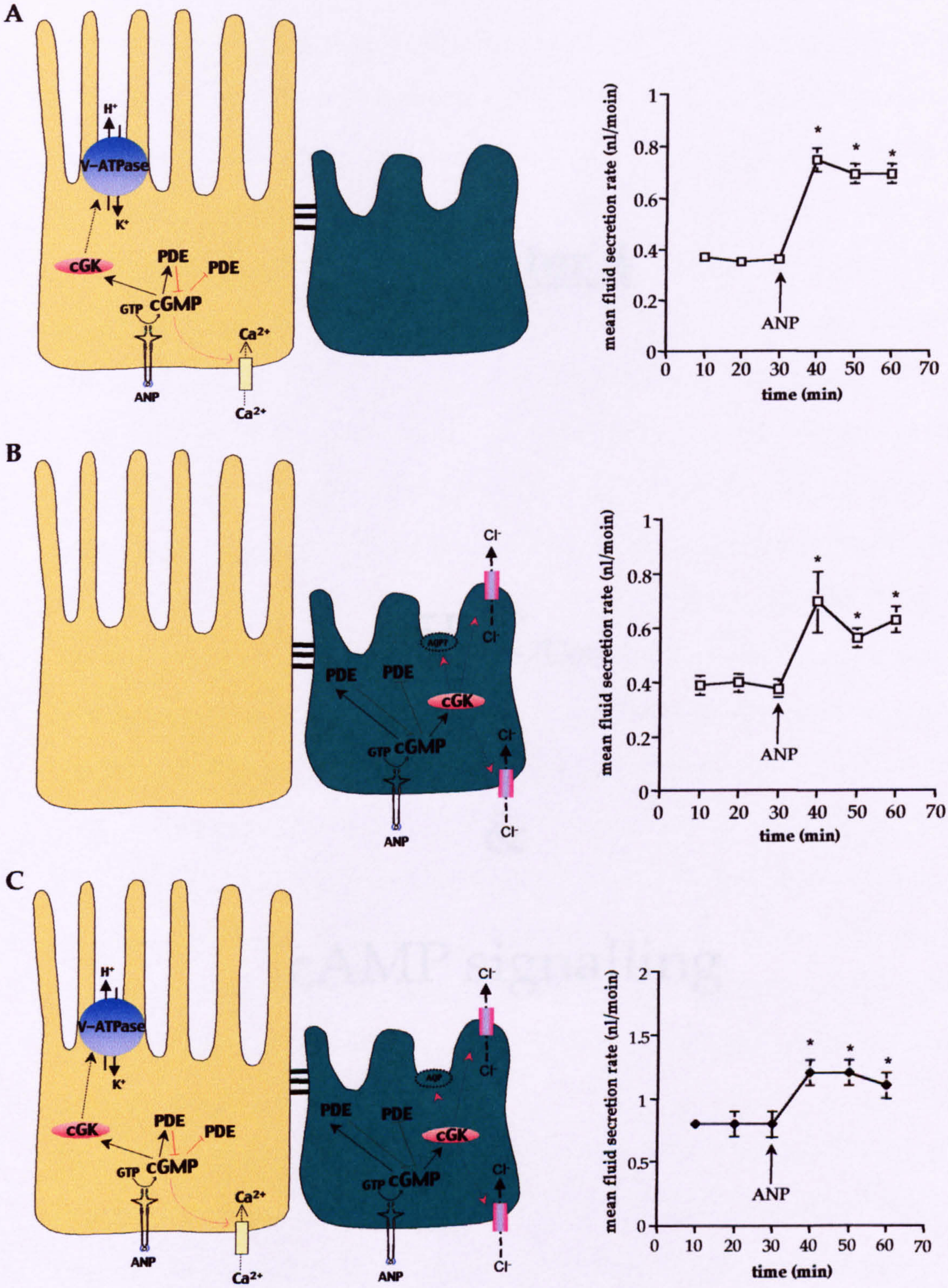


Figure 3.18: A model for cell-specific action of cGMP in GC-A transgenic Malpighian tubules

A: ANP-induced cell-specific generation of cGMP in principal cells elevates fluid secretion *via* cGMP-dependent kinase (cGK), and elevation of $[Ca^{2+}]_i$, to stimulate V-ATPase activity.

B: ANP-induced cell-specific generation of cGMP in stellate cells elevates fluid secretion *via* cGMP-dependent kinase (cGK), to increase chloride conductance.

C: ANP-induced ubiquitous generation of cGMP in Malpighian tubules has an additive effect on fluid secretion.

Chapter 4

$5HT_{7Dro}$

&

cAMP signalling

4.1 Summary

Introduction of a *Drosophila* serotonin gene ($5HT_{7Dro}$) into the genome of *Drosophila* by P-element mutagenesis creates a unique *in vivo* model to study the direct role of intracellular adenosine 3′, 5′-cyclic monophosphate ($[cAMP]_i$) in fluid secretion of Malpighian tubules. Critically, the ligand for $5HT_{7Dro}$, serotonin (5-hydroxytryptamine, 5HT), does not normally affect $[cAMP]_i$ in wild-type *Drosophila* Malpighian tubules. Expression of engineered P-element constructs pP{CaSpeR.hs::$5HT_{7Dro}$} and pP{UAS::$5HT_{7Dro}$}, allows temporal and spatial control of expression of $5HT_{7Dro}$ respectively, and transcription of the gene is demonstrated by reverse transcriptase PCR. Both fluid transport of $5HT_{7Dro}$ and elevated $[cAMP]_i$, determined by radioimmunoassay, are modulated by 5HT in a dose dependent manner in transgenic Malpighian tubules. The effect of 5HT on tubules is attenuated by the $5HT_{7Dro}$ antagonist (+)-butaclamol, at the level of cAMP production. Immunocytochemical localisation of cAMP suggests 5HT-induced elevation is confined to specific cells, as determined by the particular GAL4 driver used to express UAS::$5HT_{7Dro}$. Cell-specific cAMP-dependent phosphodiesterase activity is increased in GAL4/UAS::$5HT_{7Dro}$ treated with 5HT. cGMP–dependent phosphodiesterase activity also increases slightly in response to 5HT, even though $[cGMP]_i$ remains constant, suggesting cross talk between cAMP- and cGMP-hydrolysing phosphodiesterases.

RT-PCR analysis of tubules revealed the presence of transcripts for all *Drosophila* G protein subunits with the exception of Gβ76C, which strengthens the probabilty that elevated cAMP is achieved through activation of adenylate cyclase by $G\alpha_s$.

Finally, the use of $5HT_{7Dro}$ as a generic tool for manipulating cAMP in any tissue was illustrated by assaying intracellular levels of cAMP in brains taken from hs::$5HT_{7Dro}$ flies, both heat-shocked and non heat-shocked, in the presence and

absence of 5HT. This assay illustrates that these transgenic lines may also be used to study the effects of increased intracellular cAMP on learning and memory in *Drosophila*.

4.2 Introduction

In the preceding introduction, the role of intracellular cGMP in Malpighian tubules was discussed. Cyclic AMP was the first of the 3′-5′-nucleotides found to occur naturally and activation of cAMP controls diverse phenomena including gene transcription, metabolism and memory via several different target proteins, including ion channels, cAMP-dependent kinases and phosphodiesterases. However, its role in Malpighian tubules is less well characterised than that of the less abundant cGMP. It has been demonstrated that tubules incubated with cAMP have elevated fluid secretion rate (Coast *et al*, 1991; Maddrell, 1971; Dow *et al*, 1994b). In *Drosophila*, this response is additive to that of cGMP (Davies *et al*, 1995), the response elicited by the neuropeptide CAP_{2b} (Davies *et al*, 1995) and also to the action of the endogenous neuropeptide leucokinin, which acts to raise intracellular Ca^{2+} (Davies *et al*, 1995; O'Donnell *et al*, 1996; Terzhaz *et al*, 1999). This suggests that cAMP has a distinct signalling role in this epithelium. Furthermore, forskolin, a lipid-soluble activator of adenylate cyclase, is a potent agonist for fluid secretion in *Drosophila* (Dow *et al*, 1994b). This demonstrates that tubules have endogenous adenylate cyclase(s) and implies that cAMP is a stimulatory second messenger in tubules. It has recently been shown that a corticotropin-releasing factor-like (CRF-like) peptide (encoded by the *Dh* gene) also acts to stimulate fluid secretion rate, and that this effect is mediated by cAMP production in only principal cells, which stongly implies that the endogenous receptor for CRF-like peptide stimulates adenylate cyclase (Cabrero *et al*, submitted 2002).

The activities of adenylate cyclases are controlled dynamically by a variety of hormones, neurotransmitters and other regulatory molecules which generally activate a heterotrimeric GTP-hydrolysing protein (G protein, which consists of an α, βand γsubunit), via a seven transmembrane receptor coupled to the G protein. These G protein-coupled receptors (GPCRs) belong to a large and ancient superfamily of integral cell membrane proteins that are involved in a diverse array of signal transduction pathways, and approximately 200 genes coding for GPCRs have been identified in the *Drosophila* genome (Brody and Cravchik, 2000). Unsurprisingly, there are a number of different G protein subunit genes in the *Drosophila*, genome (8 α, 5 β, and 2 γ genes, as determined by BDGP *Drosophila* BLAST and GADFLY searches). In its inactive state, a G protein consists of a GDP bound α subunit associated with a βγ subunit. Upon activation, the α subunit exchanges GDP for GTP and dissociates from the βγ subunit. The $G\alpha_s$ subunit stimulates adenylate cyclase to catalyse the conversion of ATP to cAMP, whereas the $G\alpha_i$ inhibits adenylate cyclase. There is growing evidence that many agonists can simultaneously stimulate G proteins that activate phospholipase C (PLC) leading to the production of $InsP_3$ (with subsequent release of Ca^{2+} from internal stores) and modulate adenylate cyclase activity. Various receptors, including somatostatin (Murthy *et al*, 1996), α-2-adrenergic (Dorn *et al*, 1997) and some opioid receptors (Murthy *et al*, 1996) are reported to be negatively coupled to adenylate cyclase and concurrently positively coupled to PLC. However others, such as vasopressin and luteinising hormone receptors (Zhu *et al*, 1994) stimulate both adenylate cyclase and PLC. The strength of coupling varies from one receptor type to another and between different species.

As described in the previous chapter, a powerful method to study the role of a particular second messenger in the function of *Drosophila* Malpighian tubules is to overexpress a receptor that normally employs a specific second messenger

for intracellular signalling. In parallel with activating a specific second messenger, the ligand for the receptor must not have any endogenous effects on the tissue. Therefore, a G protein-coupled receptor that activates adenylate cyclase when stimulated with a ligand that has no endogenous action on tubules would be required.

Serotonin (5-hydroxytryptamine, 5HT) is a biogenic amine neurotransmitter found in both vertebrates and invertebrates with a large family of receptors and diverse signalling functions, such as control of sleep, appetite, pain perception and circadian rhythms. With the exception of mammalian $5HT_3$ receptors (of which there is no *Drosophila* homologue) which are ligand-gated ion channels, all 5HT receptors belong to the superfamily of G protein-coupled receptors. It has been demonstrated that although serotonin modulates fluid secretion in some insects (Morgan and Mordue, 1984; Maddrell *et al*, 1991), it does not alter cell function in *Drosophila* Malpighian tubules (Julian Dow, personal communication). A 5HT receptor positively coupled to adenylate cyclase would therefore be ideal for selective modulation of cAMP in particular tubule cell-types.

A *Drosophila* serotonin receptor was cloned from genomic and cDNA libraries by Witz *et al* (1990) using a strategy based on nucleotide sequence homology between G protein-coupled receptor genes. It comprises of one long coding sequence with no introns, which is not uncommon among genes encoding G-protein coupled receptors. This receptor is expressed predominantly in *Drosophila* heads and was found to activate adenylate cyclase in response to serotonin when stably introduced into mouse NIH 3T3 cells. At the time, this receptor exhibited highest similarity to the human 5HT1A receptor, and being the first 5HT receptor to be cloned from *Drosophila*, was named 5HT-dro. It is now apparent that this receptor has highest similarity to mammalian 5HT7 receptors and has been renamed $5HT_{7Dro}$ (Tierney, 2001; Table 1.1) and will be

referred to this way herein. This receptor gene (gift, Luc Maroteaux, Universitie L. Pasteur de Strasbourg) was subcloned into the P-element vectors pP{CaSpeR.hs/act} and pP{UAST} and inserted into the fly genome by P-element mutagenesis. Work described in this chapter aims to look at the direct role of endogenously produced intracellular cAMP in *Drosophila* Malpighian tubules using the $5HT_{7Dro}$ receptor as a modulator of intracellular cAMP levels.

4.3 Results

4.3.1 Cloning of $5HT_{7Dro}$ into P-element vectors pP{CaSpeRhs/ act} and pP{UAST}

A 1.7 kb $5HT_{7Dro}$ cDNA clone was used as a template for PCR mutagenesis to incorporate 5′ *Eco*RI and 3′ *Not*I restriction sites. The clone was amplified with a combination of *Taq* and *Pwo* DNA polymerases and subcloned into the TOPO TA cloning vector pCR2.1 (Invitrogen). The PCR product was then sequenced to verify error-free amplification, before being directionally cloned into the P-element vectors pP{CaSpeR.hs/act} and pP{UAST} using the engineered *Eco*RI and *Not*I restriction sites (Figures 4.1 and 4.2). The clone was then sequenced again using a P-element-specific primer, to confirm successful incorporation of the open reading frame. Transgenic lines created with these constructs are referred to as hs::$5HT_{7Dro}$ and UAS::$5HT_{7Dro}$ depending on which of the vectors was used.

4.3.2 Expression of $5HT_{7Dro}$ in *Drosophila melanogaster*

Transgenic lines were generated by germline transformation of embryos, as described in the preceding chapter, and inverse PCR was performed to identify the chromsomal location of the insertions. Reverse transcriptase PCR was also performed on tubules from heat-shocked hs::$5HT_{7Dro}$ flies and tubules from progeny of crosses between UAS::$5HT_{7Dro}$ lines and GAL4 lines (referred to in the text as c42/, c710/ and c724/UAS::$5HT_{7Dro}$). As before, contamination of cDNA preparations with genomic DNA was only detectable indirectly by performing PCR on tubule preparations that had not been incubated with reverse transcriptase enzyme. Figure 4.3 illustrates conditional expression of full-length transcripts from various independent lines.

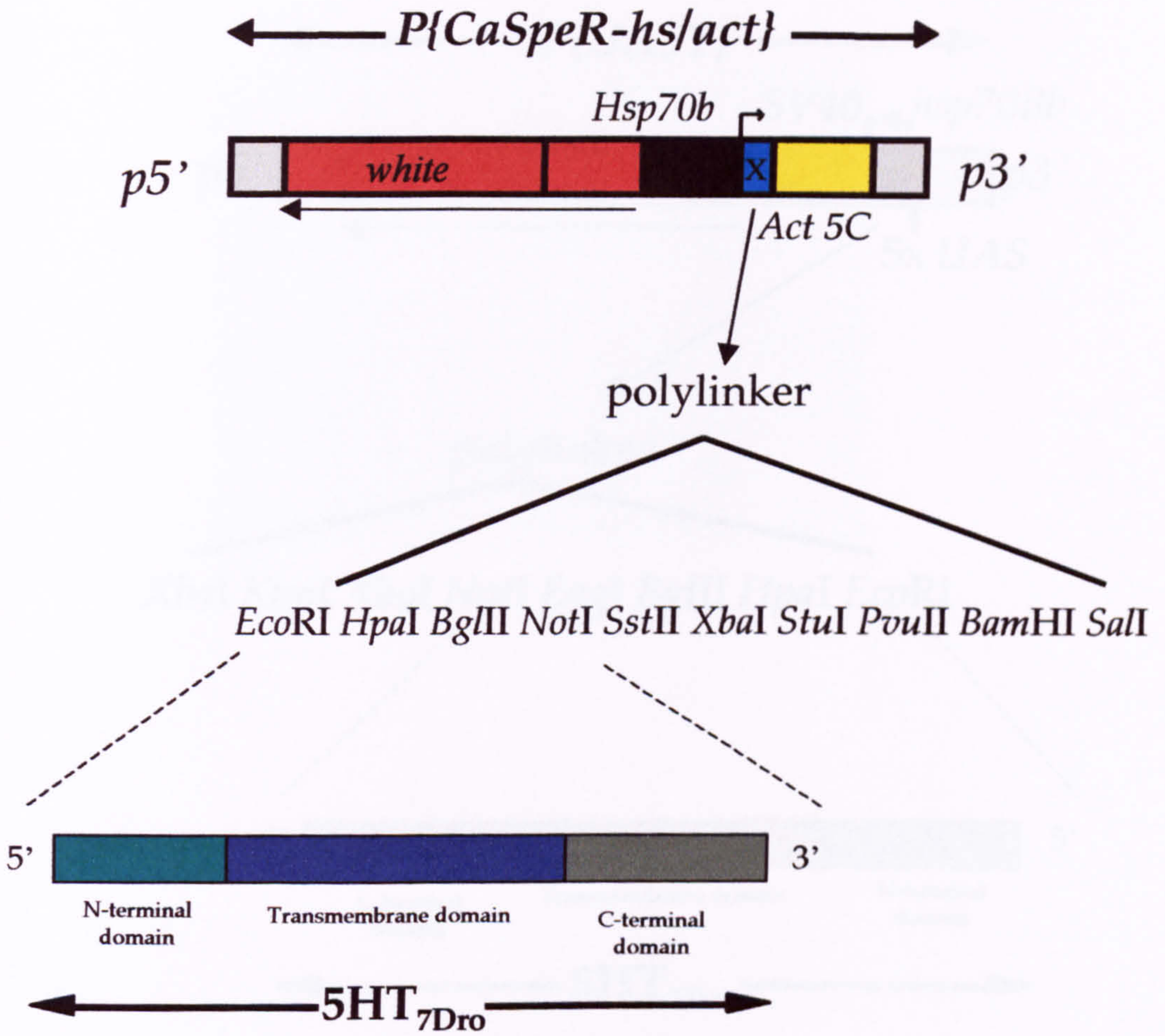


Figure 4.1: Cloning of $5HT_{7Dro}$ into pP{CaSpeR-hs/act} vector

A 1.7 kb *Drosophila* $5HT_{7Dro}$ open reading frame was amplified from a cDNA clone in pBluescript KS+. Primers used (Appendix 1) introduced 5' *Eco*RI and 3' *Not* I to the open reading frame to allow for directional cloning into pP{CaSpeRhs / act} (Thummel *et al*, 1988).

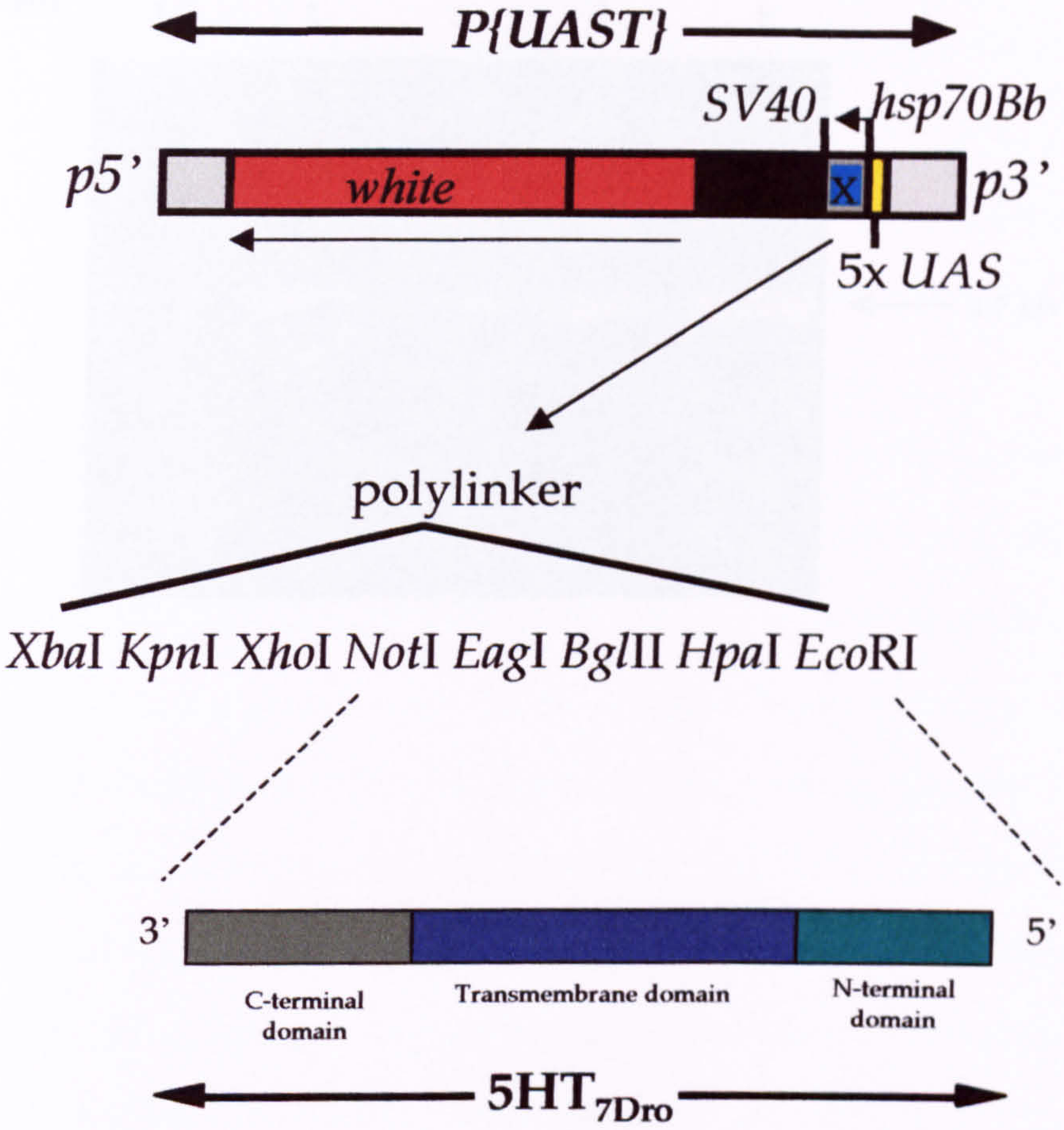


Figure 4.2: Cloning of $5HT_{7Dro}$ into pP{UAST} vector

A 1.7 kb *Drosophila* $5HT_{7Dro}$ open reading frame was amplified from a cDNA clone in pBluescript KS+. Primers used (Appendix 1) introduced 5' *Eco*RI and 3' *Not* I to the open reading frame to allow for directional cloning into pP{UAST} (Brand and Perrimon, 1993).

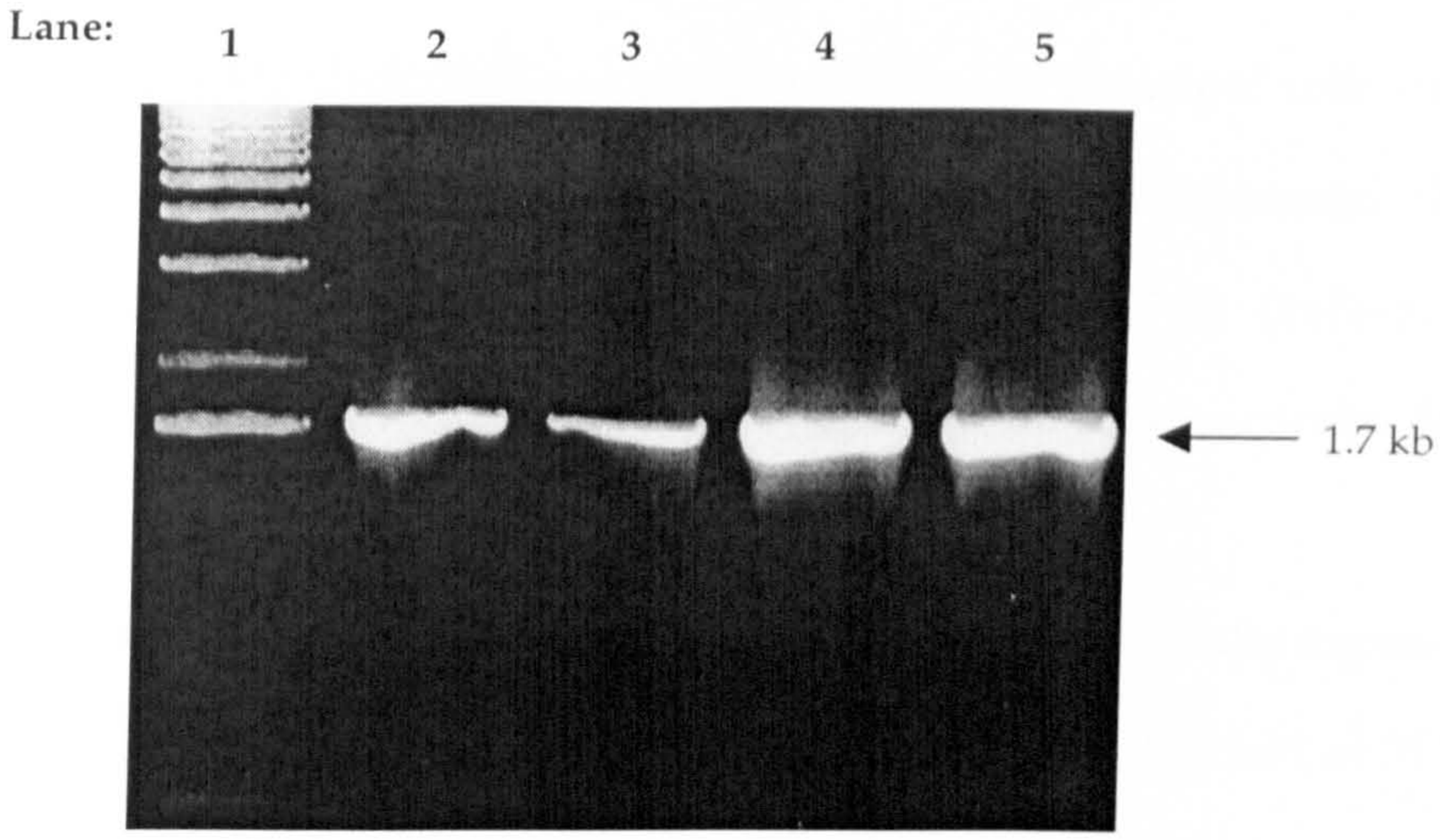


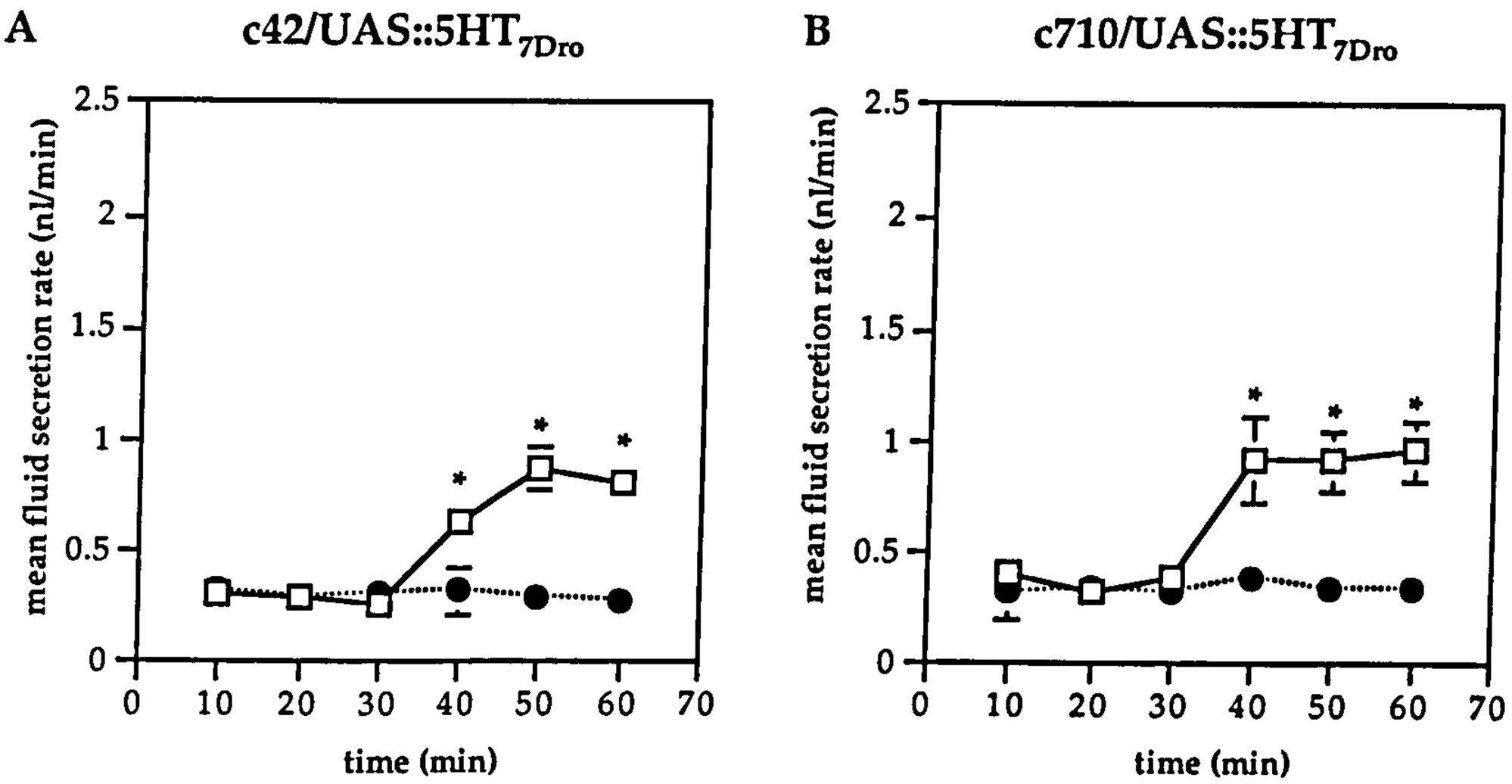
Figure 4.3: The expression of $5HT_{7Dro}$ in transgenic lines

cDNA from tubules expressing $5HT_{7Dro}$ was used as template for RT PCR. Lane 1: 1 kb DNA ladder (Gibco BRL). Lane 2: c710/UAS:: $5HT_{7Dro}$ cDNA as template. Lane 3: c724/UAS:: $5HT_{7Dro}$ cDNA as template. Lane 4: c42/UAS:: $5HT_{7Dro}$ cDNA as template. Lane 5: hs:: $5HT_{7Dro}$ cDNA from heat-shocked flies as template. No expression of $5HT_{7Dro}$ was detected with cDNA from non heat-shocked hs::$5HT_{7Dro}$ flies as template (not shown). No genomic products were detected in an RT-PCR using intron spanning primers for the *vha*55 gene (not shown). This indicated that cDNA samples did not have genomic contamination, and that products detected with primers for $5HT_{7Dro}$ were not generated from genomic DNA.

4.3.3 Conditional expression of $5HT_{7Dro}$ in tubules confers 5HT-induced elevation of fluid transport.

Progeny of a cross between the strain pP{UAS::$5HT_{7Dro}$} and GAL4 line c42 (referred to as c42/UAS::$5HT_{7Dro}$) express $5HT_{7Dro}$ in principal cells of the main segment of tubules. Similarly, progeny of a cross between the strain pP{UAS::$5HT_{7Dro}$} and GAL4 lines c724 or c710 (referred to as c724/UAS::$5HT_{7Dro}$ and c710/UAS::$5HT_{7Dro}$ respectively) express $5HT_{7Dro}$ in stellate cells of tubules.

Flies from the strain pP{CaSpeRhs::$5HT_{7Dro}$} which conditionally express $5HT_{7Dro}$ under heat-shock (referred to as hs::$5HT_{7Dro}$) were heat-shocked at 37°C for 45 min 12-18 h before use. Tubules from all the flies above were then dissected for both fluid secretion assays and cAMP assays. Fluid secretion rate was measured for 30 min before 5HT was added to the bathing solution. The fluid secretion rate was measured for a further 30 min. All $5HT_{7Dro}$ tubules displayed sensitivity to serotonin (5HT). Figure 4.4A shows a typical fluid secretion assay on c42/UAS::$5HT_{7Dro}$ flies and Figure 4.4B. shows a typical fluid secretion assay on c710/ and c724/UAS::$5HT_{7Dro}$ flies. The maximum rate of fluid secretion achieved in heat-shocked hs::$5HT_{7Dro}$ tubules in response to 5HT is additive to those achieved when the $5HT_{7Dro}$ is expressed in principal or stellate cells only (Figure 4.4C). Calculations of fluid secretion rate were made as described in the previous chapter. In all tubules expressing the $5HT_{7Dro}$ receptor, there was a significant elevation of fluid secretion rate above basal, which was sustained over 30 min.



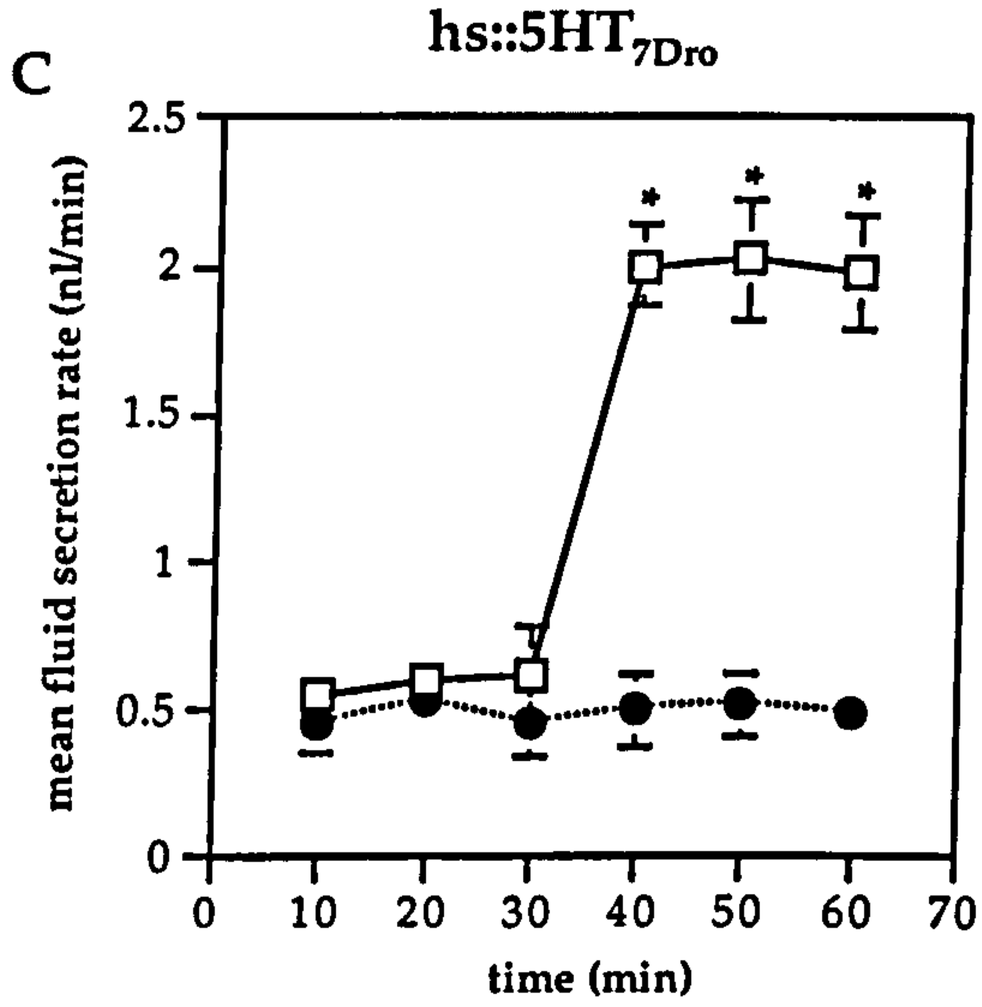


Figure 4.4: Expression of $5HT_{7Dro}$ transgene confers 5HT-stimulated elevation of fluid transport

Basal secretion rates were measured for 30 min before tubules were treated with 1μM 5HT. Secretion rates were measured for a further 30 min.

A: Tubules from c42/UAS::$5HT_{7Dro}$ flies (□) show elevated fluid secretion rates in response to 5HT, n=10. Tubules from UAS::$5HT_{7Dro}$ (●) do not respond to 5HT, n=10.

B: Tubules from c710/UAS::$5HT_{7Dro}$ flies (□) show elevated fluid secretion rates in response to 5HT, n=10. Tubules from UAS::$5HT_{7Dro}$ (●) do not respond to 5HT, n=10.

C: Tubules from heat-shocked hs::$5HT_{7Dro}$ flies (□) show elevated fluid secretion rates in response to 5HT, n=10. Tubules from non heat-shocked hs::$5HT_{7Dro}$ flies (●) do not respond to 5HT, n=10., n=10.

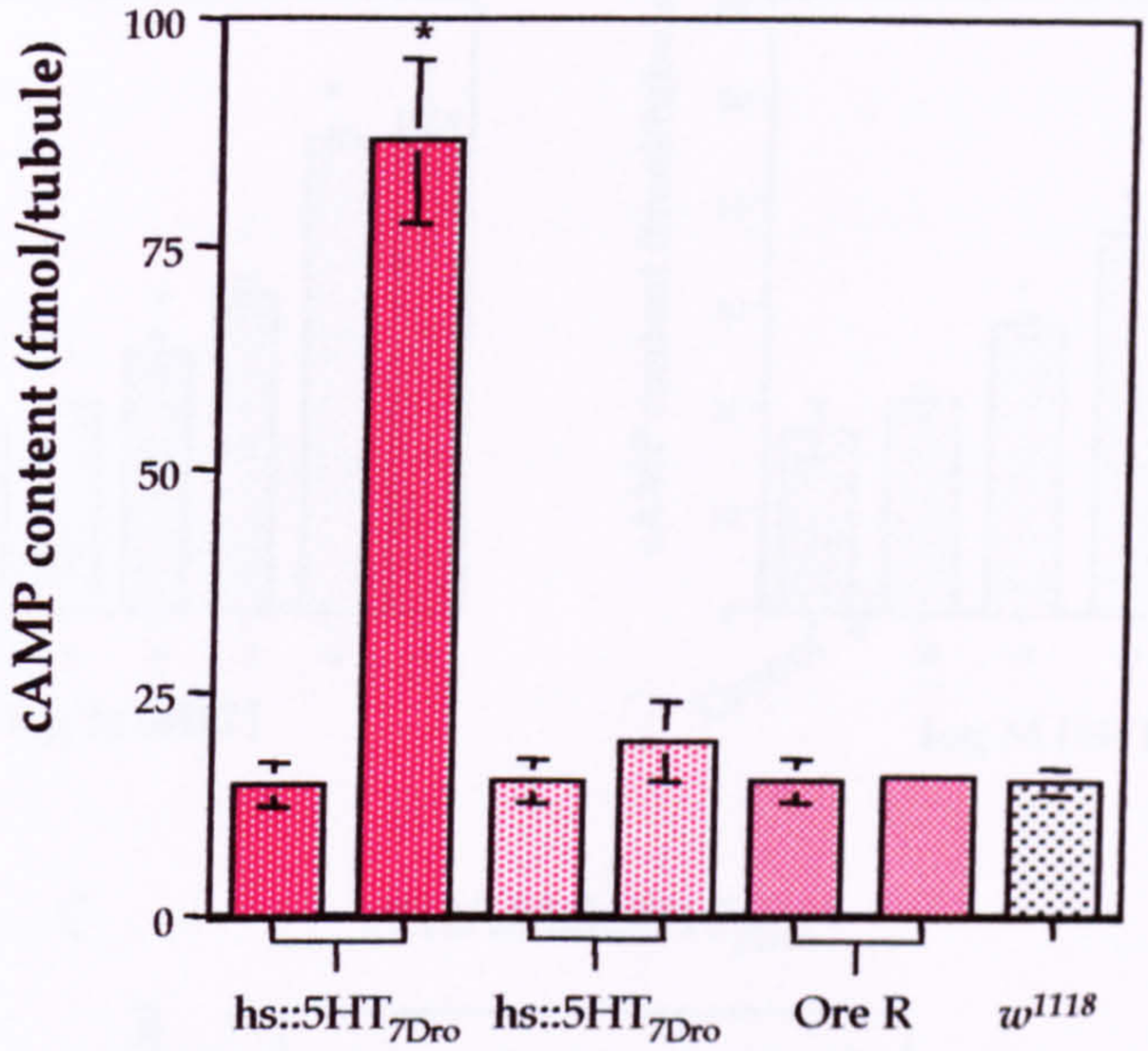
Data shown are expressed as mean fluid secretion rate (nl/min) ± SEM.

Secretion rates significantly different from basal are denoted by *. ($P<0.05$, determined with the Student's *t*-test, on unpaired samples, assuming unequal variances).

4.3.4 5HT-stimulated tubules expressing the $5HT_{7Dro}$ transgene elevate intracellular cAMP levels.

Cyclic AMP levels were assayed in tubule samples from heat-shocked hs::$5HT_{7Dro}$ flies with either absence or presence of 1 μM 5HT. These samples were compared to cAMP levels in tubule samples from non heat-shocked hs::$5HT_{7Dro}$ flies with either absence or presence of 1 μM 5HT and control samples from heat-shocked wild type flies treated with 1 μM 5HT (Figure 4.5). All samples showed similar intracellular levels of cAMP (approximately 250 fmol/tubule), with the exception of the heat-shocked hs::$5HT_{7Dro}$ sample which had been treated with 5HT. This sample contained 82 ±5 fmol (approximately fourfold cAMP content), indicating that the $5HT_{7Dro}$ receptor had been stimulated and the associated G protein α subunit had upregulated adenylate cyclase activity, a cAMP hydolysing phosphodiesterase activity had been downregulated, or that modulation of both these enzymes had resulted in elevated $[cAMP]_i$.

The expression of the $5HT_{7Dro}$ gene was also targetted to specific cell types in the tubule by crossing UAS::$5HT_{7Dro}$ flies with c42, c724 and c710 GAL4 enhancer trap lines (Figure 4.6). Tubules from progeny of these crosses were sensitive to 5HT, as reflected by the elevation of intracellular cAMP. Furthermore, the quantity of intracellular cAMP generated was directly proportional to the concentration of 5HT used in the assay, with a calculated mean EC_{50} for production of cAMP of 2.1×10^{-8} M ($\pm 0.7 \times 10^{-8}$ M), consistent with published data (Witz *et al*, 1990). Tubules with $5HT_{7Dro}$ localised to principal cells gave rise to larger increases in cAMP than tubules expressing $5HT_{7Dro}$ in stellate cells, which reflects the greater volume and number of principal cells in tubules. Nevertheless, despite their smaller size and number, stellate cells were still able to produce a large amount of cAMP in response to 5HT. Control tubules, lacking transgenic $5HT_{7Dro}$, showed no elevation of cAMP in response to 5HT.



Heat shock	+	+	-	-	+	+	+
5HT	-	+	-	+	-	+	+

Figure 4.5: 5HT stimulates cAMP production in heat-shocked hs::5HT$_{7Dro}$ but not in wild-type tubules

Flies were heat-shocked at 37°C for 1hr 12-18 hours prior to use. Tubules were treated with 1 μM 5HT and the phosphodiesterase inhibitor IBMX (10^{-4}M) for 10 min, and then prepared as described in Materials and Methods. Results are expressed as mean cAMP content (fmol/tubule) ± SEM, n=3. cAMP content significantly different from basal cAMP content is denoted *. ($P<0.05$, as determined with Student's *t*-test on unpaired samples, assuming unequal variances).

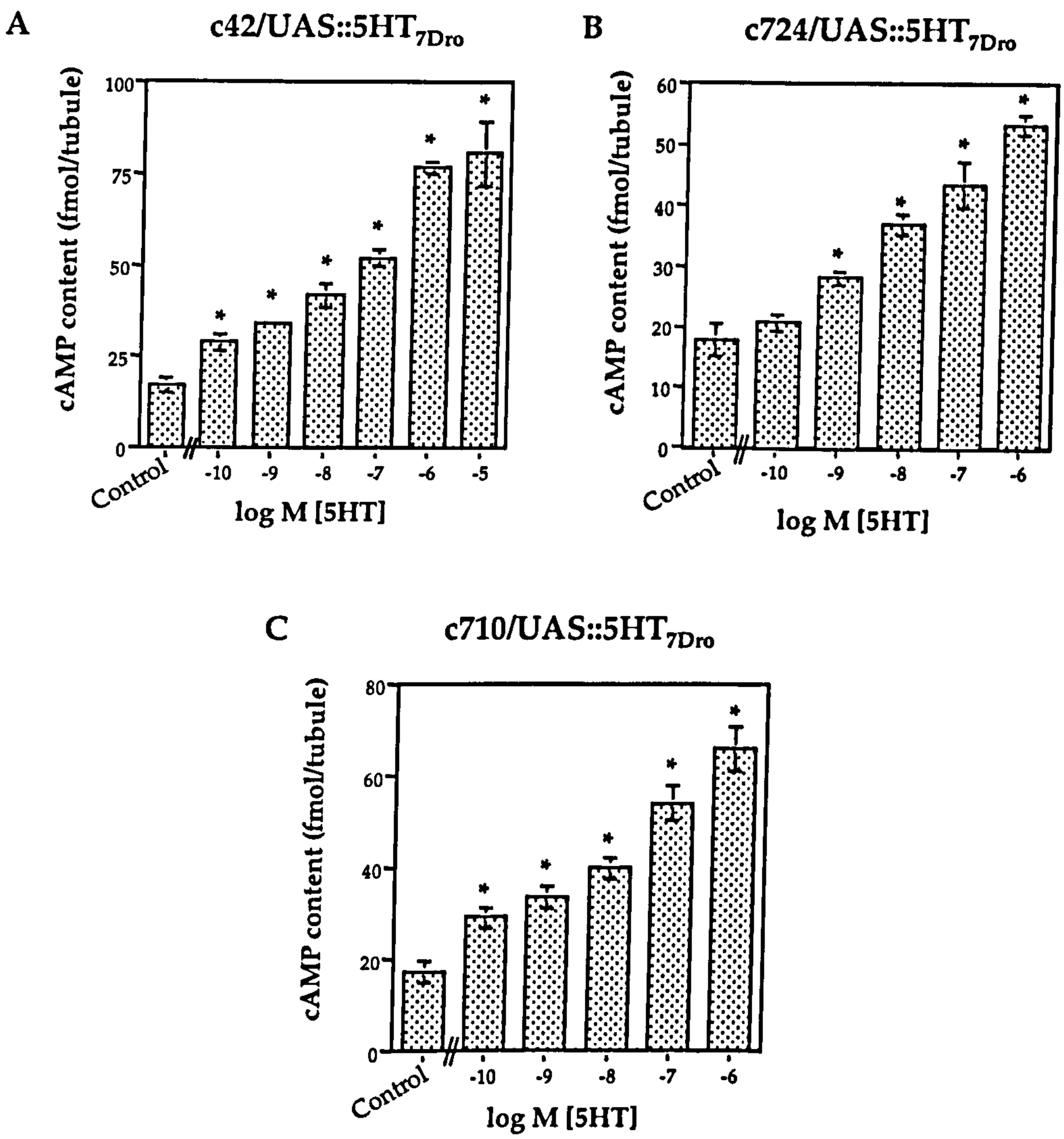


Figure 4.6 : cAMP content in UAS::5HT$_{7Dro}$ transgenic tubules is elevated by 5HT in a dose dependent manner

Tubules were incubated with 5HT and the phosphodiesterase inhibitor IBMX (10^{-4} M) for 10 minutes. Control samples were prepared in the absence of 5HT.

A: Tubules from progeny of a cross with the principal cell-specificGAL4 driver line c42 and a UAS::5HT$_{7Dro}$ line, n≥5.

B: Tubules from progeny of a cross with the stellate cell-specificGAL4 driver line c724 and a UAS::5HT$_{7Dro}$ line, n=6.

C: Tubules from progeny of a cross with the stellate cell-specificGAL4 driver line c710 and a UAS::5HT$_{7Dro}$ line, n≥4.

Results are expressed as mean cAMP content (fmol/tubule) ± SEM. cAMP content significantly different from basal cAMP content is denoted by *. ($P<0.05$, determined with Student's *t*-test on unpaired samples, assuming unequal variances).

4.3.5 (+)-butaclamol inhibits 5HT-induced fluid secretion and cAMP production in $5HT_{7Dro}$ transgenic tubules.

The addition of the competitive inhibitor (+)-butaclamol to heat-shocked hs::$5HT_{7Dro}$ transgenic tubules was shown to attenuate the 5HT-induced rise in fluid secretion (Figure 4.7A). The production of cAMP in tubules co-presented with (+)-butaclamol (at various concentrations) and 1 μM 5HT was assayed using tubules from the progeny of a cross between UAS::$5HT_{7Dro}$ flies and the principal cell specific GAL4 enhancer trap line c42. There was a dose dependent attenuation of cAMP production in these samples, with 2.5 x 10^{-8} M (± 1 x 10^{-8} M) (+)-butaclamol required to achieve half-maximal inhibition of serotonin activity (Fig 4.7B). Interestingly, 5HT-induced cAMP production, in tubules treated with 10^{-5} M (+)-butaclamol, was attenuated by 75% of control 5HT-stimulated transgenic $5HT_{7Dro}$ tubule cAMP content. This was consistent with fluid secretion data, whereby tubules, treated with 10^{-5} M (+)-butaclamol, showed 5HT-induced elevated fluid secrtion rates that were approximately 25% of the elevated fluid secretion rates observed in 5HT-stimulated control tubules.

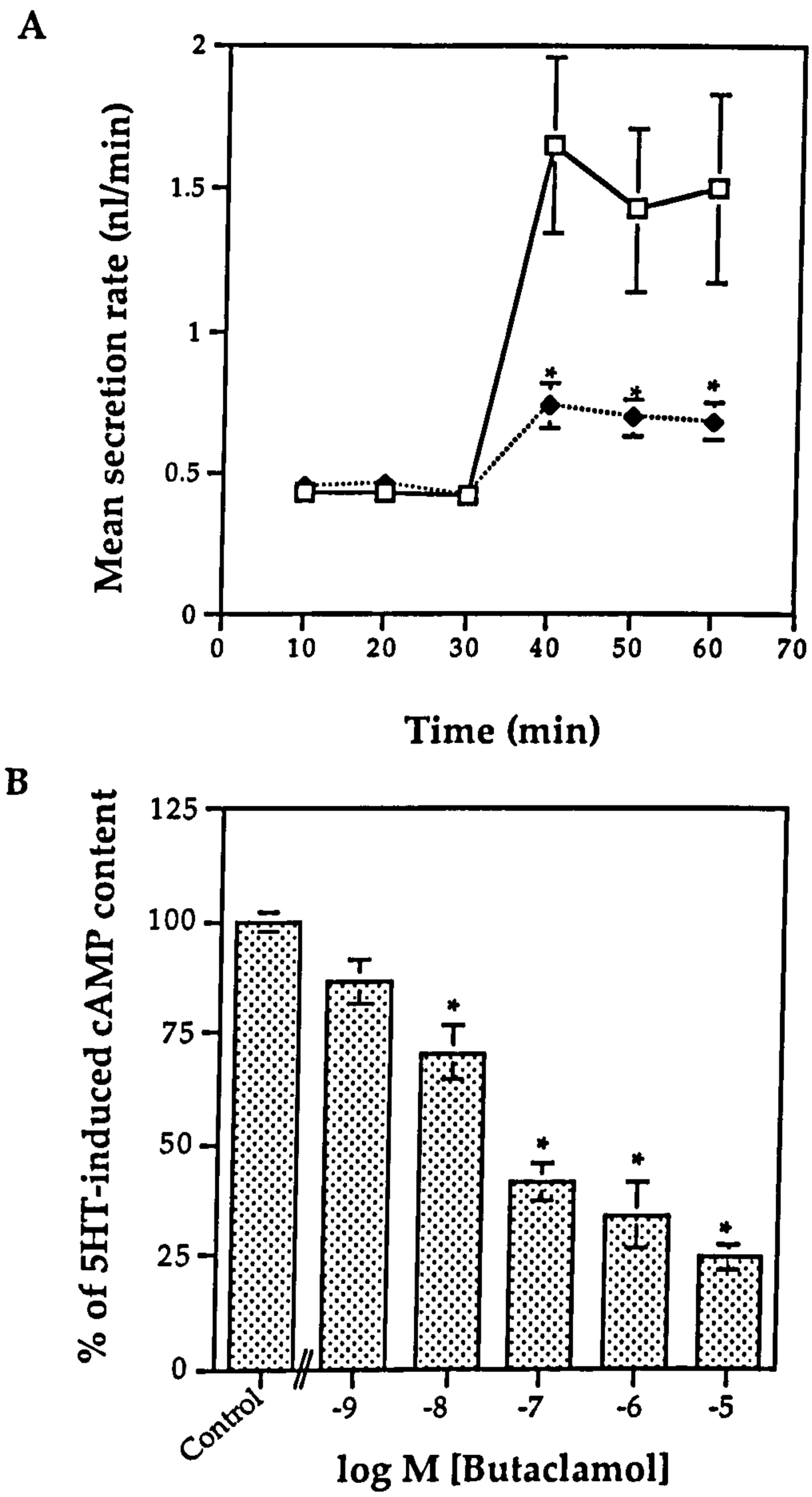


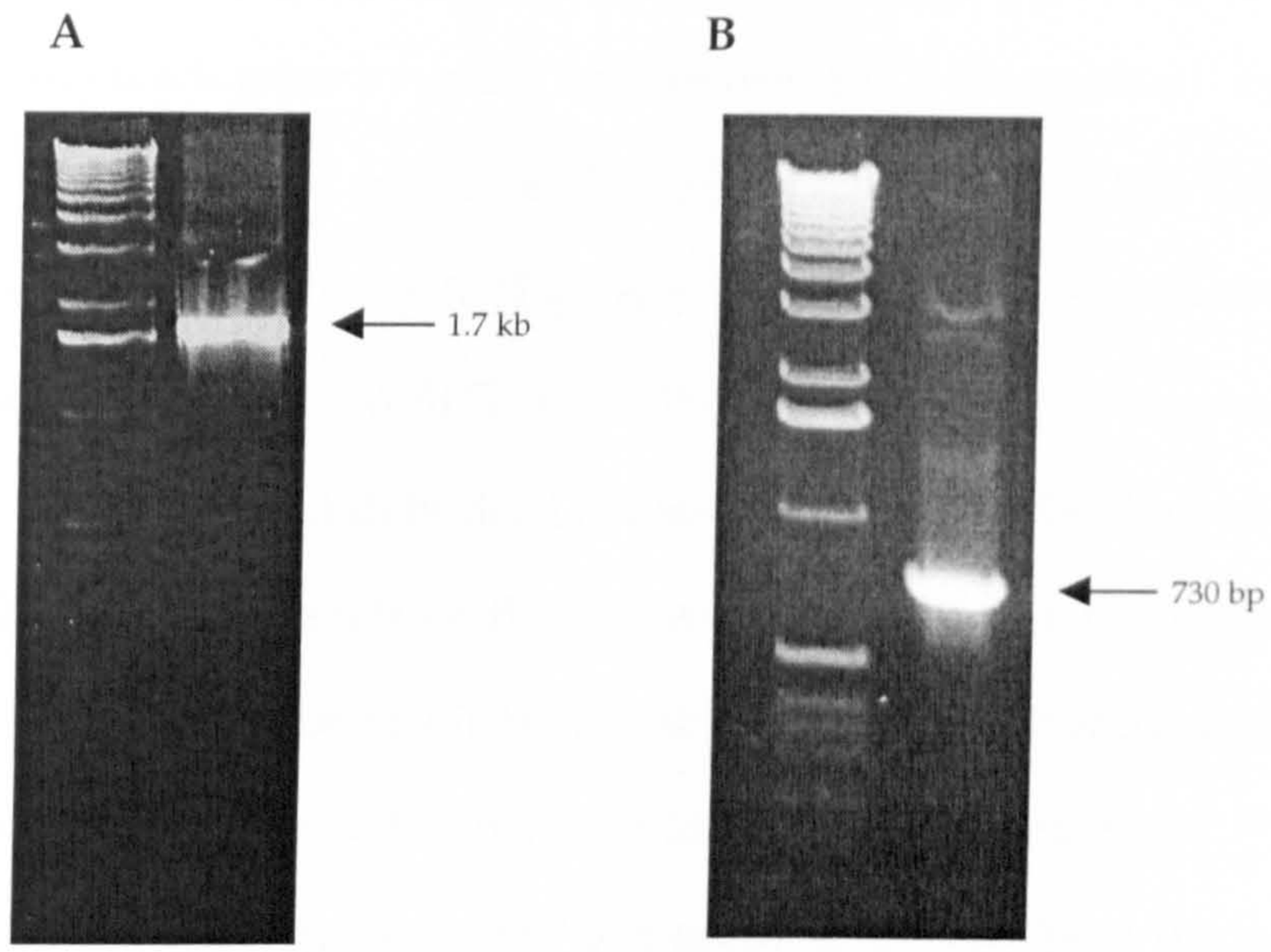
Figure 4.7: (+)-Butaclamol inhibits 5HT-induced fluid secretion and cAMP elevation in $5HT_{7Dro}$ transgenic tubules

A: Heat-shocked hs::$5HT_{7Dro}$ tubules challenged with 1 μM 5HT at 30 min showed elevated fluid secretion rates (□) Tubules treated with the 5HT antagonist (+)-butaclamol (10^{-5} M) at t=0 showed an attenuated response to 5HT (◆). Results shown are mean secretion rate (nl/min) ± SEM, n=10.
Secretion rates significantly different from control, 5HT-treated, rates (□) are denoted by *. ($P<0.05$, determined with the Student's *t*-test, on unpaired samples, assuming unequal variances).

B: Tubules from progeny of a cross with c42 (principal cell GAL4 driver line) and UAS:: $5HT_{7Dro}$ were treated with 1μM 5HT, the phosphodiesterase inhibitor IBMX (10^{-4} M) and varying concentrations of the $5HT_{7Dro}$ antagonist (+)-butaclamol for 10 minutes. A control sample untreated with (+)-butaclamol Tubules were then prepared as described in Materials and Methods. Results are expressed as percentages of cAMP content in 5HT-stimulated tubules unchallenged with (+)-butaclamol ± SEM, n=3. cAMP content significantly different from maximal cAMP content is denoted by *. ($P<0.05$, determined with Student's *t*-test on unpaired samples, assuming unequal variances).

4.3.6 Generation of a UAS::$5HT_{7Dro}$ / GFP fusion construct.

A commercial antibody to the $5HT_{7Dro}$ receptor was not available to study localisation of the receptor in tubules, so a fusion construct allowing detection of the receptor with green fluorescent protein was made. The $5HT_{7Dro}$ gene and GFP open reading frame were amplified from plasmids using a combination of *Taq* and *Pwo* polymerases (Figure 4.8 A and B). These PCR fragments were amplified with primers which introduced novel restriction sites at the 5′ end of $5HT_{7Dro}$ and 3′ end of GFP and primers with overlapping sequence (10 bases) at the 3′ end of $5HT_{7Dro}$ and 5′ end of GFP. The PCR fragments were then gel purified, mixed together and fused in a PCR reaction that did not have additional primers. The outcome of this PCR reaction was one product consisting of the $5HT_{7Dro}$ gene with GFP tagged on to the 3′ end (Figure 4.8 C). The PCR fragment was then subcloned into pUAST and sequenced to verify successful tagging. The protein product would have GFP fused to the C-terminal end of $5HT_{7Dro}$, which would not affect ligand binding or G-protein interaction. Due to time constraints this construct was not used to germline transform flies, but remains a plausible alternative to antibody generation to localise this receptor.



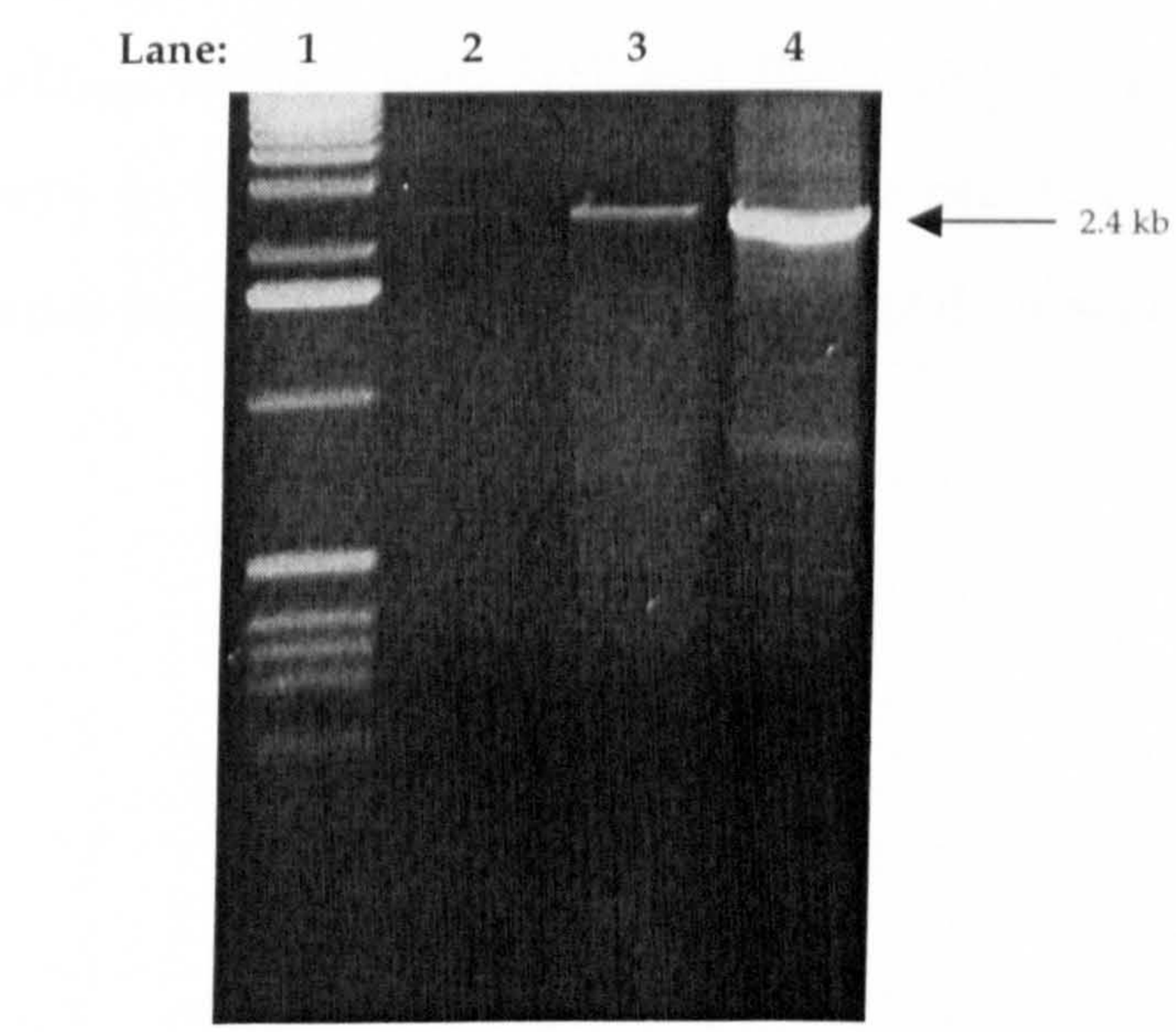


Figure 4.8: Fusion of $5HT_{7Dro}$ and GFP by PCR

$5HT_{7Dro}$ and GFP were amplified from plasmids with primers which introduced novel restriction sites at the 5'end of $5HT_{7Dro}$ and 3' end of GFP and had overlapping sequence by 10 bases at the 3' end of $5HT_{7Dro}$ and 5' end of GFP (A and B) (Appendix 1). These PCR products were gel purified, mixed together and fused by PCR amplification in the absence of primers, with the minimum number of PCR cycles. Five, ten and twenty cycle reactions were carried out (C: lanes 2, 3 and 4 respectively). The resulting PCR product thus has the GFP open reading frame fused to the C-terminal of $5HT_{7Dro}$(C). The PCR product from lane 3 (C) was then cloned into pUAST to allow for conditional expression in *Drosophila*.

4.3.7 Immunocytochemical localisation of cAMP.

The localisation of cAMP generated in $5HT_{7Dro}$ transgenic tubules was detected by immunocytochemistry using antiserum to 2'-O-succinyl cyclic AMP conjugated to BSA raised in rabbit (Figure 4.9 and Figure 4.10). Tubules from progeny of crosses with UAS::$5HT_{7Dro}$ and GAL4 lines were treated with IBMX (10^{-4} M) alone or with 1 μM 5HT and IBMX (10^{-4} M) for ten min before being fixed with 4 % paraformaldehyde. Tubules treated with IBMX alone displayed very low background levels of fluorescence with the fluorescein-conjugated anti-rabbit secondary antibody (data not shown). Tubules treated with 5HT and IBMX showed high levels of fluorescence in a cell-specific manner.

In c42/UAS::$5HT_{7Dro}$ tubules, cAMP appeared to be localised throughout the principal cells of the main segment (Figure 4.9), but appeared to have strongest localisation in pools at the basolateral membrane and nuclei (Figure 4.9). Stellate cell-specific generation of cAMP was achieved by stimulating c724/UAS::$5HT_{7Dro}$ tubules with 5HT and IBMX (Figure 4.10). Cyclic AMP localised mainly to these cells, and similar to the c42/UAS::$5HT_{7Dro}$ tubules, there appeared to be particularly intense perinuclear fluorescence.

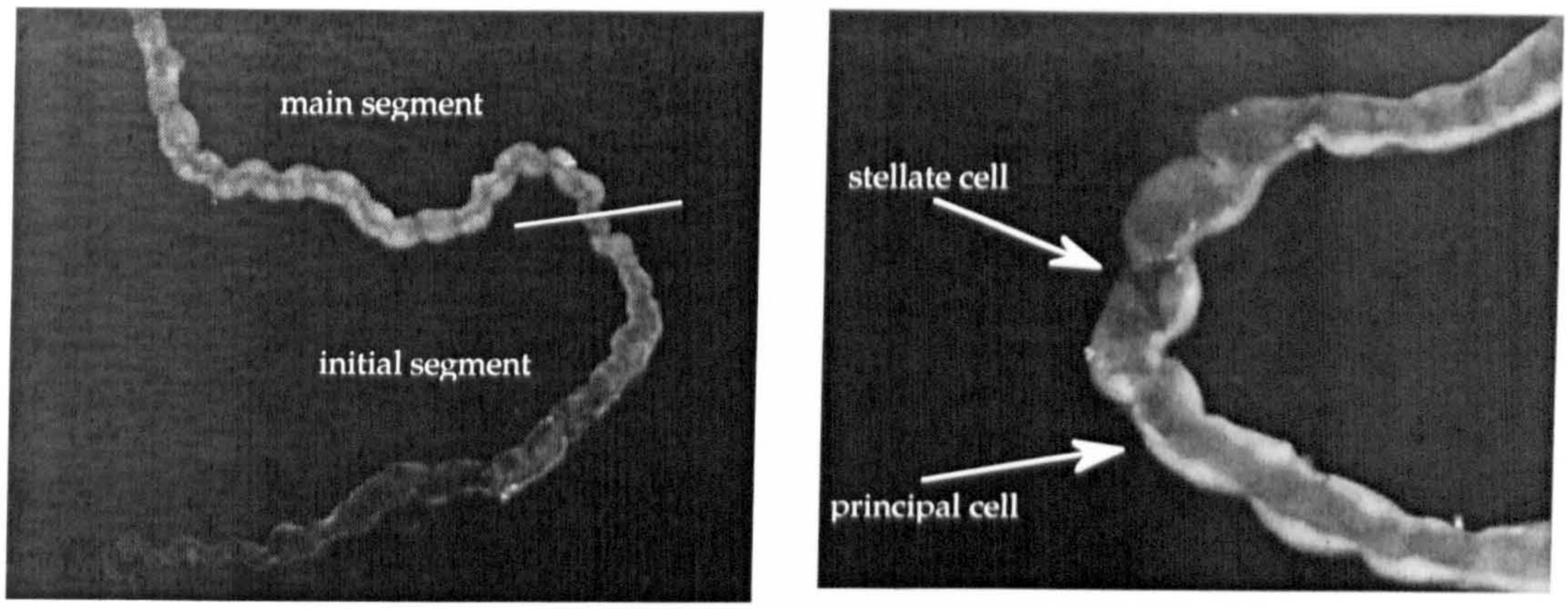


Figure 4.9: Anti-cAMP immunocytochemical analysis reveals cell-specific generation of cAMP in c42/UAS::5HT$_{7Dro}$ transgenic tubules in response to 5HT

Tubules from c42/UAS::5HT$_{7Dro}$ lines were treated with the phosphodiesterase inhibitor IBMX (10^{-4}M) and 1 μM 5HT for 10 minutes before being fixed, as described in Materials and Methods. Tubules were incubated with rabbit anti-cAMP antiserum and then with fluorescein-conjugated anti-rabbit second antibody before being viewed under UV light. A: Cyclic AMP production is confined to principal cells in the main segment of tubules (top half) (100 x magnification). B: Cyclic AMP production forms pools at the basolateral membrane of principal cells but is not elevated in stellate cells (arrow) (200 x magnification).

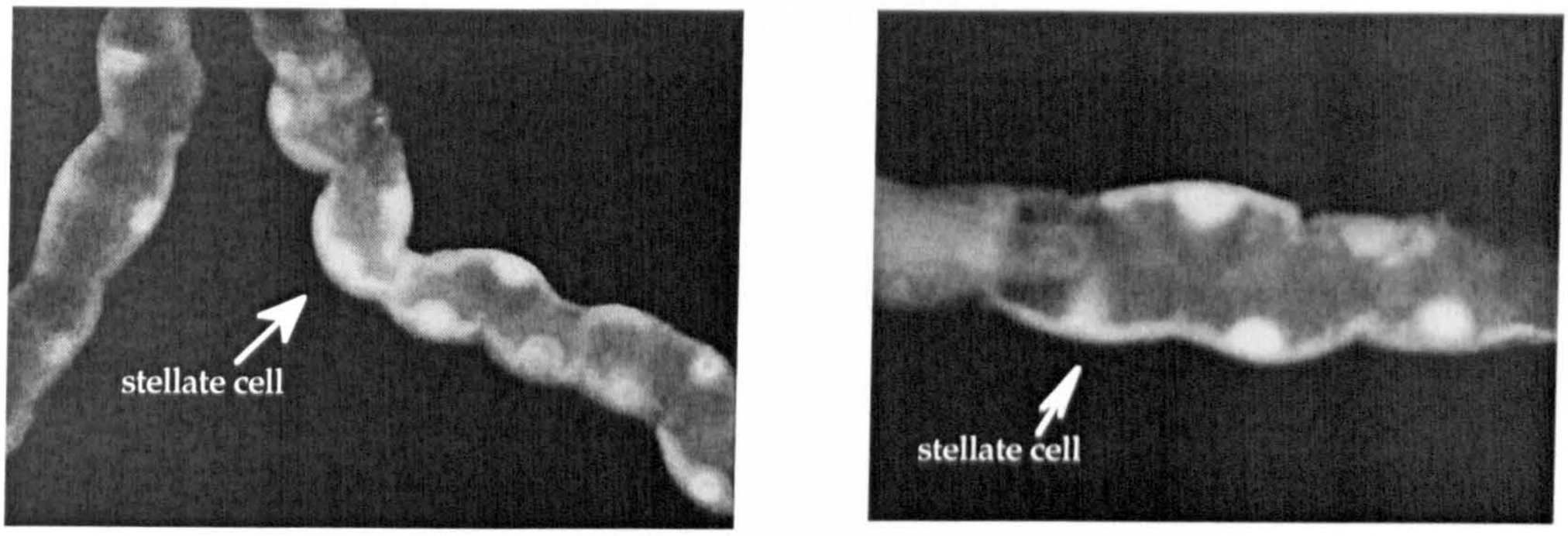


Figure 4.10: Anti-cAMP immunocytochemical analysis reveals cell-specific generation of cAMP in c724/UAS::5HT$_{7Dro}$ transgenic tubules in response to 5HT

Tubules from c724/UAS::5HT$_{7Dro}$ lines were treated with the phosphodiesterase inhibitor IBMX (10^{-4} M) and 1 μM 5HT for ten minutes before being fixed, as described in Materials and Methods. Tubules were incubated with rabbit anti-cAMP antiserum and then with fluorescein-conjugated anti-rabbit second antibody before being viewed under UV light. A: Cyclic AMP production is confined to stellate cells of tubules (200 x magnification). B: 400 x magnification of the main segment. Stellate cells have elevated cAMP content after incubation with 5HT, which appears throughout the cell.

4.3.8 Cyclic GMP levels in $5HT_{7Dro}$ transgenic tubules

The possibility that intracellular cGMP levels may be altered upon activation of $5HT_{7Dro}$ was investigated by assaying levels both with and without 5HT stimulation in samples from $5HT_{7Dro}$ expressing tubules and from a wild type control (Figure 4.11). All samples contained approximately the same basal levels of intracellular cGMP, and there was no significant change in these levels upon 5HT-induced $5HT_{7Dro}$ activation. This demonstrates that cAMP is the specific downstream effector of $5HT_{7Dro}$ and that the effects on cell function do not include generation or inhibition of synthesis of cGMP.

4.3.9 $[Ca^{2+}]_i$ in 5HT-stimulated $5HT_{7Dro}$ transgenic tubules

Intracellular Ca^{2+} levels were assayed in tubules from progeny of a cross between male UAS::$5HT_{7Dro}$ flies and female UAS::apoaequorin;+/+;c42 flies for principal cell-specific measurements and a cross between male UAS::$5HT_{7Dro}$ flies and female UAS::apoaequorin;+/+;c710 flies for stellate cell-specific measurements. Tubules responded to 5HT with a rapid elevation of $[Ca^{2+}]_i$ in principal cells followed by a sustained secondary response (Figure 4.12A). Cross-talk is therefore taking place between cAMP or a cAMP-dependent effector and mechanisms dependent on Ca^{2+} in principal cells.

There was no change in $[Ca^{2+}]_i$ in stellate cells, suggesting that no cross-talk occurs in these cells (Figure 4.12B).

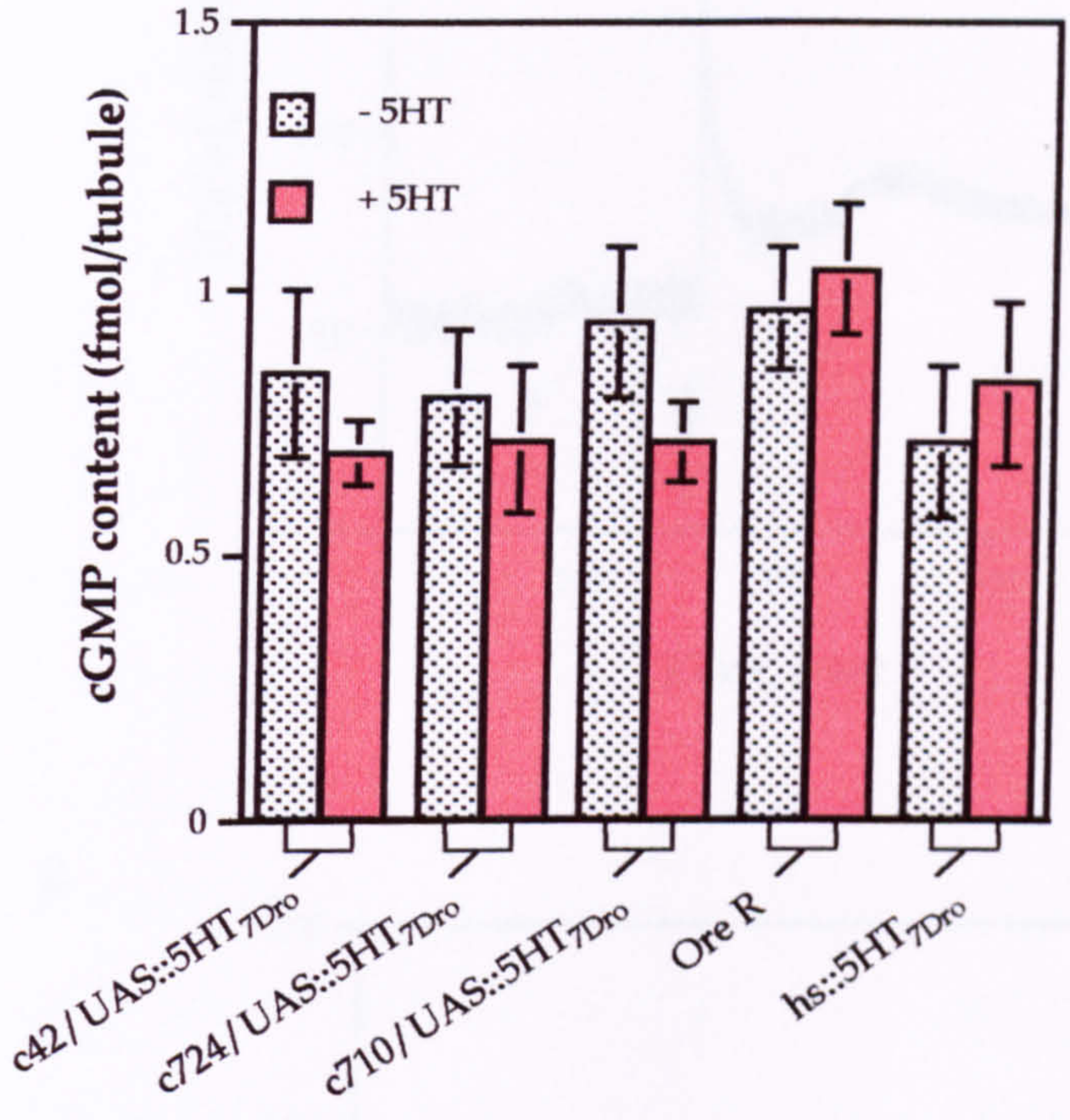
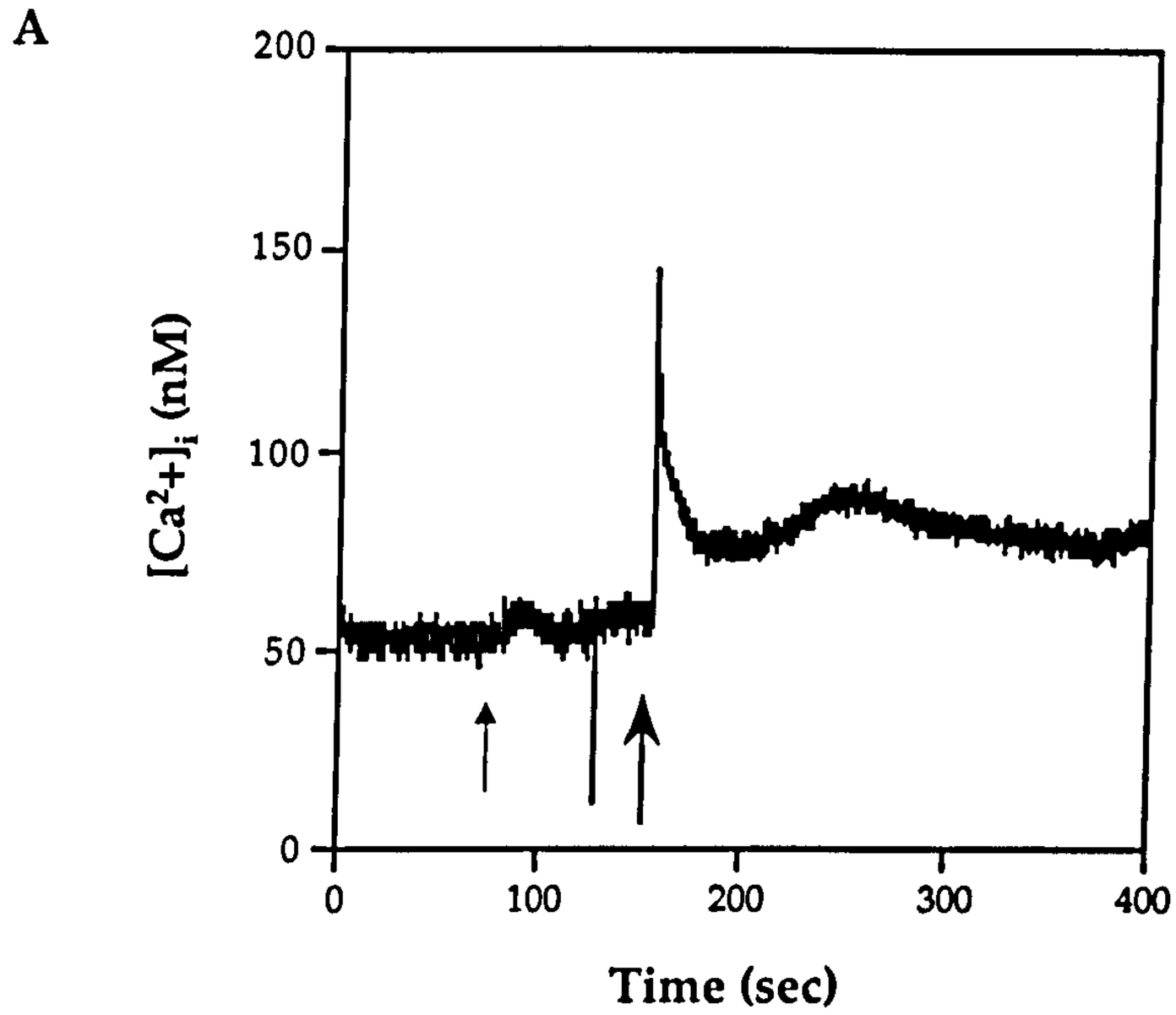


Figure 4.11 : $5HT_{7Dro}$ transgenic tubules do not have significantly altered cGMP levels in response to 5HT

Tubules were treated with 5HT (1 μM) and Zaprinast (1 μM) for 10 min and prepared as in Materials and Methods. Results are expressed as mean cGMP content (fmol/tubule) ± SEM, n=3. Differences between samples 5HT-treated samples and control samples are not significant ($P>0.05$, determined with Student's *t*-test on unpaired samples, assuming unequal variances).



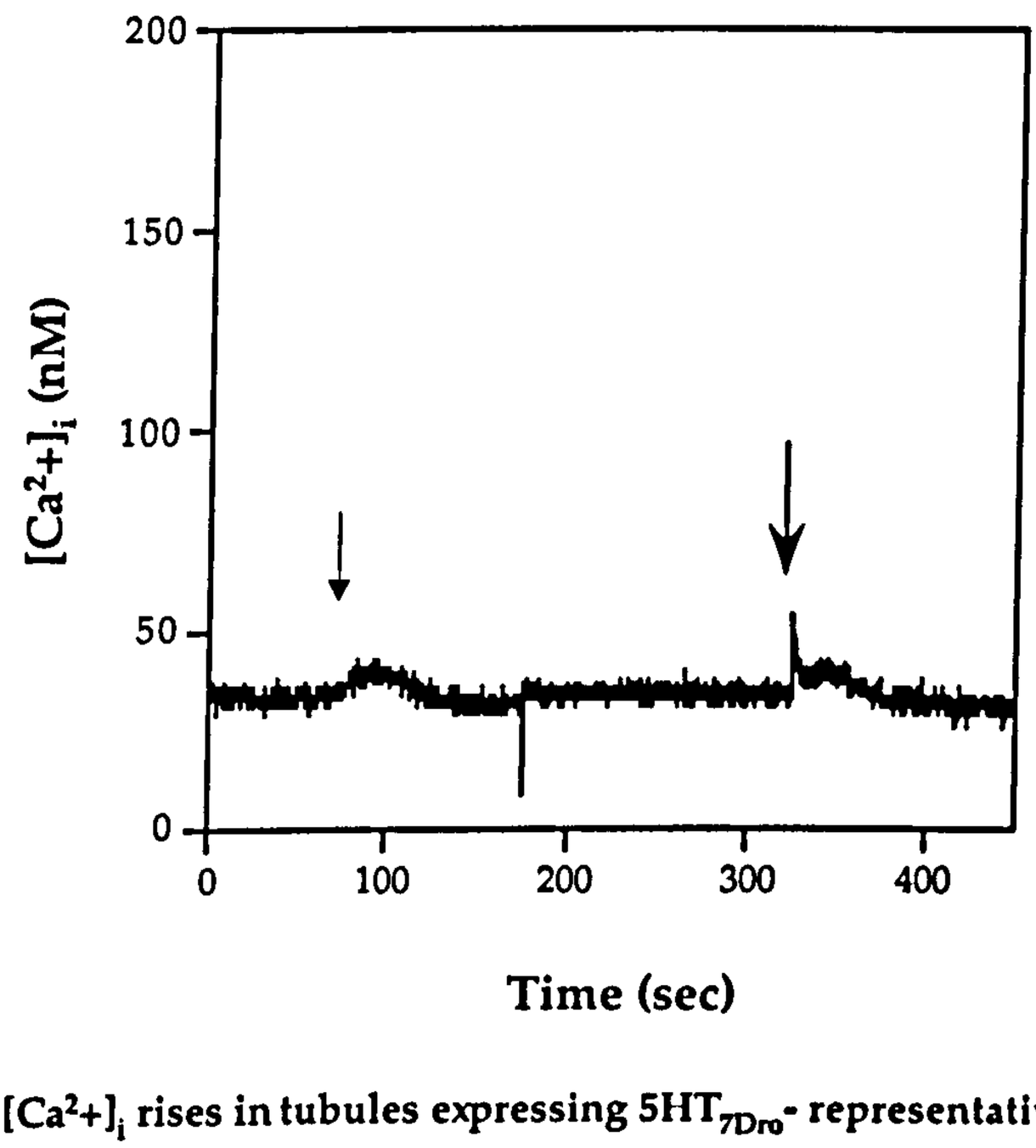


Figure 4.12: 5HT-induced $[Ca^{2+}]_i$ rises in tubules expressing $5HT_{7Dro}$- representative traces

Typical traces of changes in $[Ca^{2+}]_i$ in tubule cells expressing $5HT_{7Dro}$ when stimulated with $1x10^{-8}$ M 5HT (large arrows).

A: Ca^{2+} response to 5HT in principal cells of aeq;+/+; c42 GAL4/$5HT_{7Dro}$ tubules.

B: Ca^{2+} response to 5HT in stellate cells of aeq; +/+; c710 GAL4/$5HT_{7Dro}$ tubules.

The peaks prior to the main rises in $[Ca^{2+}]_i$ are caused by control ("mock") injections of Schneider's medium (small arrows).

4.3.10 Cell-specific phosphodiesterase activity in $5HT_{7Dro}$ transgenic flies

Tubules expressing $5HT_{7Dro}$ in a cell-specific manner were treated with 5HT for 10 min, then transferred to PDE buffer. Tubules expressing $5HT_{7Dro}$ in principal cells showed an approximate twofold increase in cAMP-hydrolysing phosphodiesterase activity, suggesting that the signal achieved by a large rise in cAMP in these cells is quickly dampened by elevation of phosphodiesterase activity. Tubules expressing $5HT_{7Dro}$ in stellate cells also showed increased cAMP-hydrolysing phophodiesterase activity upon stimulation with 5HT (Figure 4.13A). Activity of cGMP-hydrolysing phosphodiesterases in samples, both stimulated and untreated with 5HT, was also measured (4.13B). There appeared to be increased cGMP-hydrolysing phosphodiesterase activity in 5HT-stimulated samples, which suggests that there was an element of cross-talk between phosphodiesterases to achieve this up-regulation, or that a single cAMP-regulated phosphodiesterase has dual substrate specificity. Interestingly, in the previous chapter it was shown that upregulation of cGMP-hydrolysing phosphodiesterase activity in GC-A transgenic tubules by ANP was concurrent with a decrease in cAMP-hydrolysing phosphodiesterase activity (Figure 3.16B), with no significant change in intracellular cAMP levels (Figure3.13).

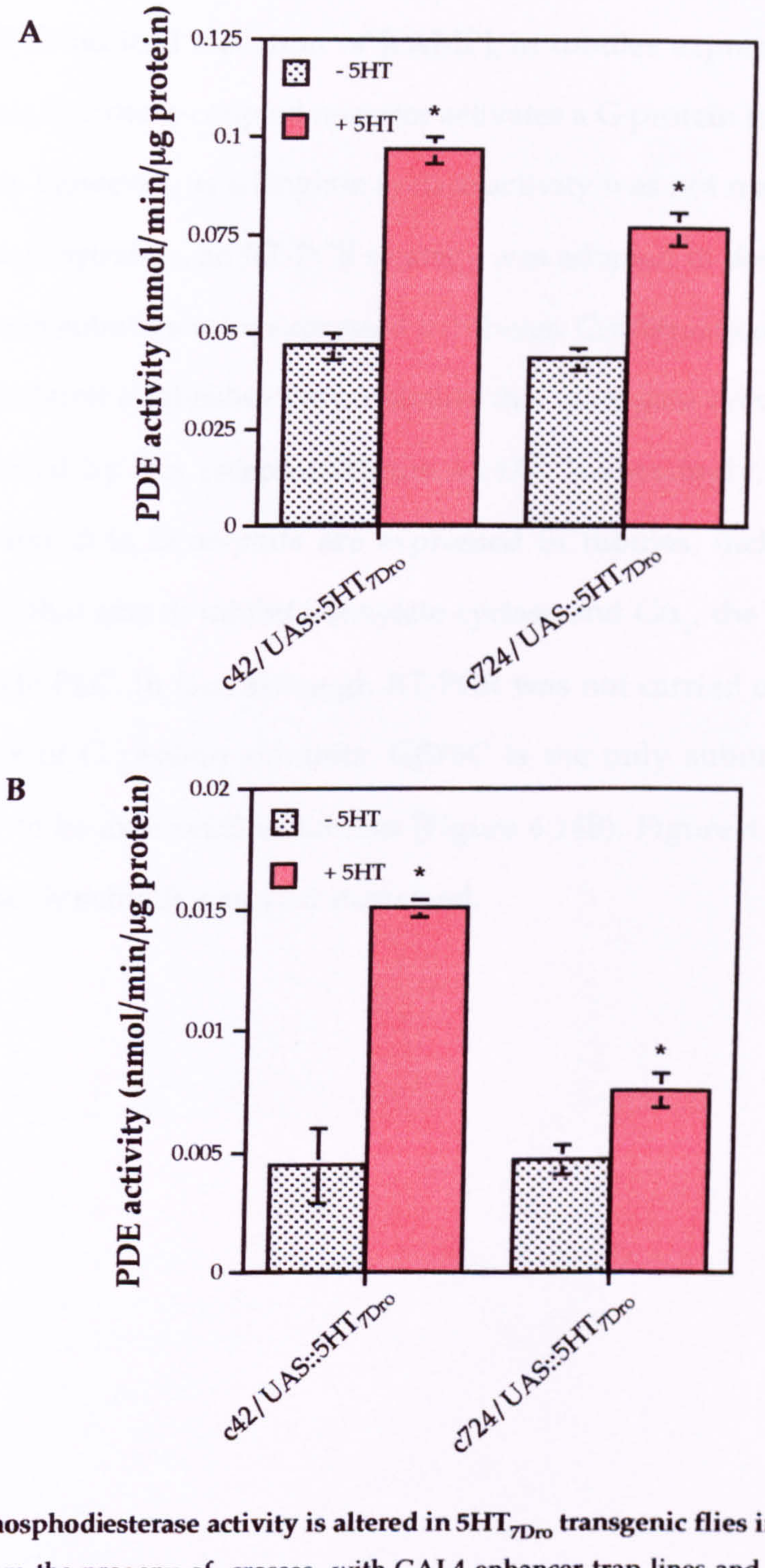


Fig 4.13: Phosphodiesterase activity is altered in $5HT_{7Dro}$ transgenic flies in response to 5HT

Tubules from the progeny of crosses with GAL4 enhancer trap lines and UAS::$5HT_{7Dro}$ lines were treated with 1 µM 5HT for 10 minutes. Phosphodiesterase activity was assayed as described in Materials and Methods.

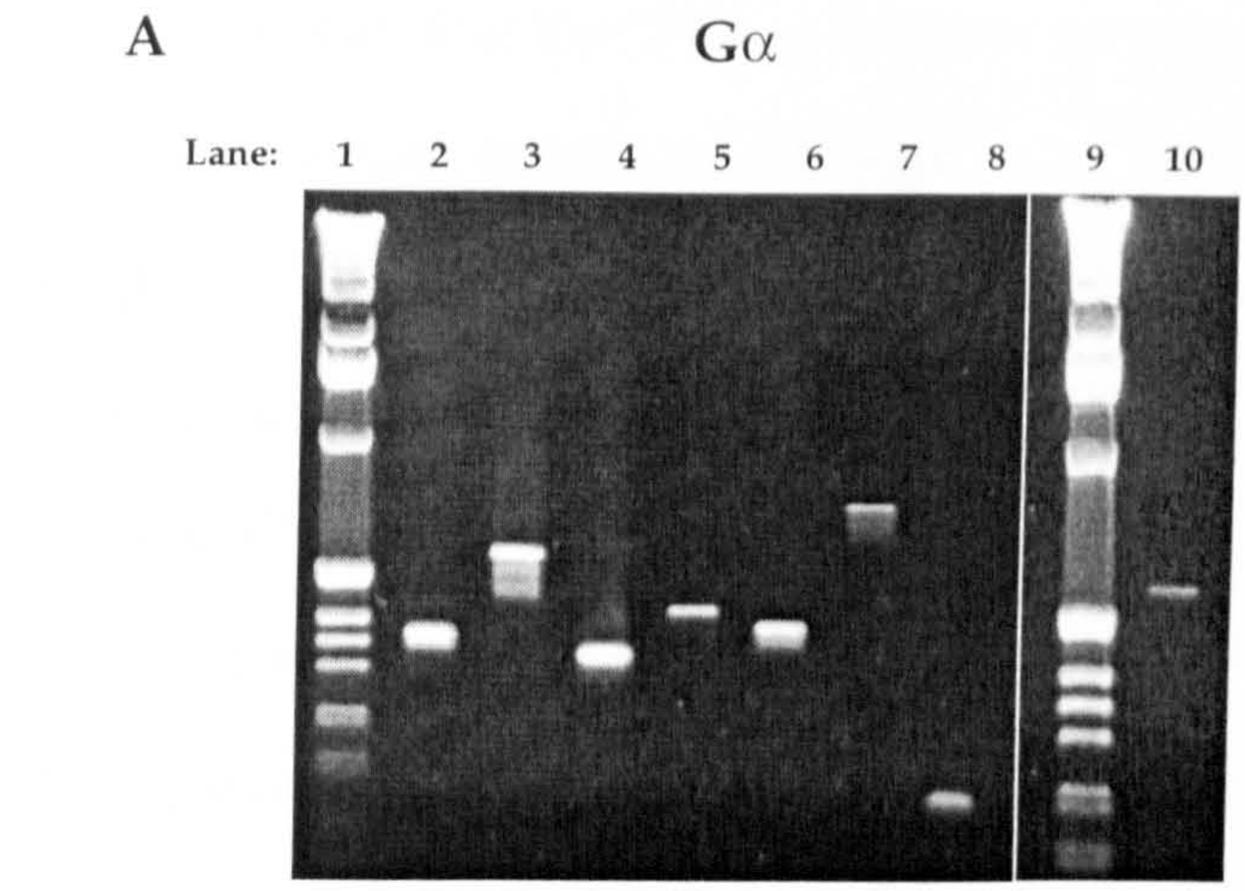
A: Samples were assayed for cAMP-dependent phosphodiesterase activity.

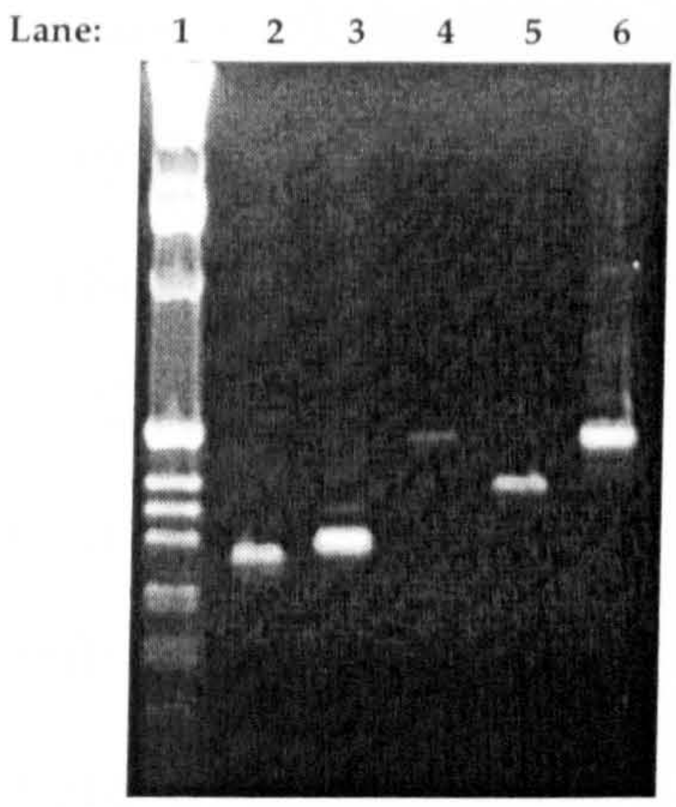
B: Samples were assayed for cGMP-dependent phosphodiesterase activity.

Results are expressed as mean phosphodiesterase activity (nmol/min/µg) ± SEM, n=3. PDE activity significantly different from basal PDE activity is denoted by *. ($P<0.05$, determined with Student's *t*-test on unpaired samples, assuming unequal variances).

4.3.11 Expression of G protein subunits in Malpighian tubules

The 5HT-induced elevation of $[cAMP]_i$ in tubules expressing $5HT_{7Dro}$ implies that this G protein-coupled receptor activates a G protein to stimulate adenylate cyclase. However, as adenylate cyclase activity was not measured directly, due to time constraints, an RT-PCR strategy was adopted to determine which of the G protein subunits were expressed in tubules. Confirmation of the expression of a $G\alpha_s$ subunit establishes the possibility that adenylate cyclase activity could be modulated by this receptor (Figure 4.14A). Interestingly, all the Gα subunit genes found in *Drosophila* are expressed in tubules, including $G\alpha_i$, the Gα subunit that acts to inhibit adenylate cyclase and $G\alpha_q$, the subunit that acts to stimulate PLC. In fact, although RT-PCR was not carried out for all the splice variants of G protein subunits, Gβ76C is the only subunit which does not appear to be expressed in tubules (Figure 4.14B). Figure 4.14C illustrates that both the Gγ subunit genes are expressed.





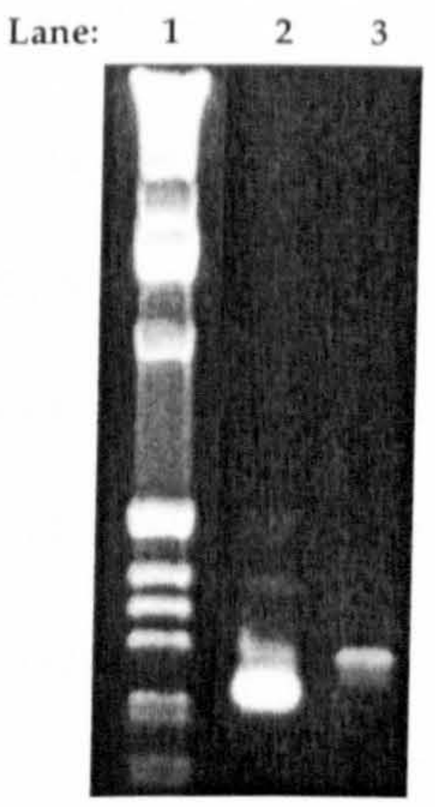


Figure 4.14: The expression of G protein subunits in Malpighian tubules

Reverse transcriptase polymerase chain reaction (RT-PCR) using wild-type tubule template cDNA with intron-flanking primers directed against each *Drosophila* G protein subunit.

A: G protein alpha (G_α) subunits.

Lane 1: 1 kb ladder (Gibco BRL). Lane 2: $G\alpha_s$60A, product of expected 357 bp. Lane 3: $G\alpha_i$65A, product of expected 554 bp. Lane 4: Gα73B, product of expected 326 bp. Lane 5: Gα2B, product of expected 409 bp. Lane 6: Gα *(cta)*, product of expected 384 bp. Lane 7: $G\alpha_o$47A, product of expected 705 bp. Lane 8: GαCG17760, product of expected 104 bp. Lane 9: 1 kb ladder (Gibco BRL). Lane 10: Gα49B, product of expected 575 bp.

B: G protein beta (Gβ) subunits.

Lane 1: 1 kb ladder (Gibco BRL). Lane 2: Gβ60B, product of expected 277 bp. Lane 3: Gβ8F, product of expected 303 bp. Lane 4: Gβ76C, no product of expected size (233 bp). Lane 5: Gβ5F, product of expected 407 bp. Lane 6: Gβ13F, product of expected 513 bp.

C: G protein gamma (Gγ) subunits.

Lane 1: 1 kb ladder (Gibco BRL). Lane 2: Gγ1, product of expected 252 bp. Lane 3: Gγ30A, product of expected 295 bp.

4.3.12 $[Ca^{2+}]_i$ in cAMP-stimulated $5HT_{7Dro}$ transgenic tubules

There is the possibilty that the $5HT_{7Dro}$ receptor promiscuously associates with a G protein from the $G\alpha_q$ family, to influence $[Ca^{2+}]_i$ in 5HT-stimulated tubules. Therefore, $[Ca^{2+}]_i$ levels in cAMP-stimulated tubules were assayed, to determine if cAMP could directly influence Ca^{2+} signalling.

Intracellular Ca^{2+} levels were assayed in cAMP-stimulated tubules from UAS::apoaequorin;+/+;c42 GAL4 flies, for principal cell-specific measurements and UAS::apoaequorin;+/+;c710 GAL4 flies, for stellate cell-specific measurements. A slow elevation in $[Ca^{2+}]_i$ was seen in principal cells, similar to that seen in cGMP-stimulated tubules, but no change in stellate cell $[Ca^{2+}]_i$ occurred (Figure 4.15). As stellate cells are known to express $G\alpha_q$ subunit(s) (determined by the action of the leucokinin G protein-coupled receptor *via* $G\alpha_q$ on Ca^{2+} signalling (Terhzaz *et al*, 1999; Radford *et al*, 2002)), it is likely that the 5HT-induced Ca^{2+} signal generated in principal cells of c42/$5HT_{7Dro}$ tubules is downstream of elevated cAMP, rather than direct Ca^{2+} stimulation by $G\alpha_q$. This does not confirm that the $5HT_{7Dro}$ receptor only associates with its cognate $G\alpha$ subunit, but implies that this is so.

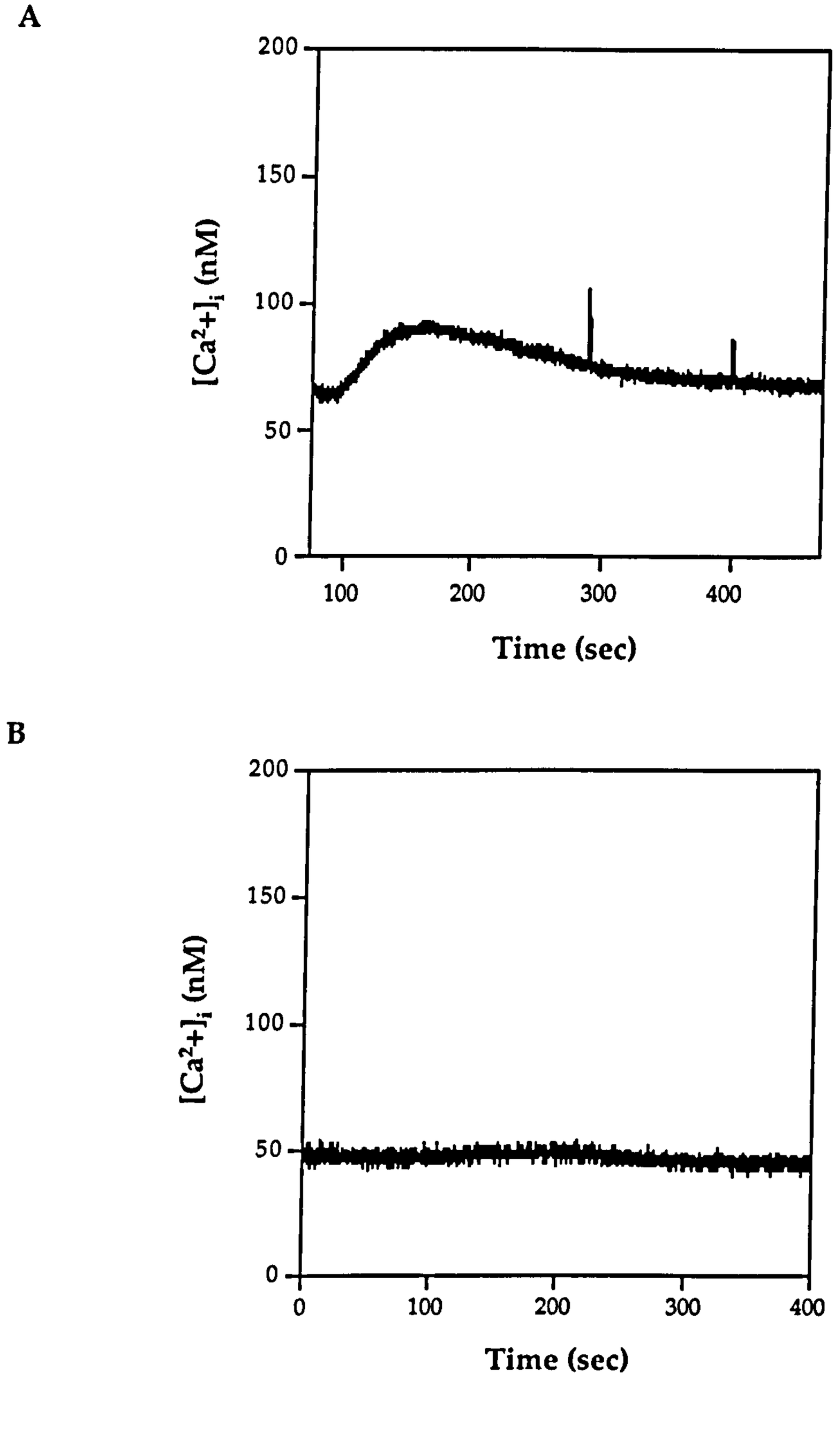


Figure 4.15: cAMP-induced $[Ca^{2+}]_i$ rises in tubules expressing $5HT_{7Dro}$- representative traces

Typical traces of changes in $[Ca^{2+}]_i$ in tubule cells expressing $5HT_{7Dro}$ when stimulated with $1x10^{-4}$ M cAMP (large arrows).

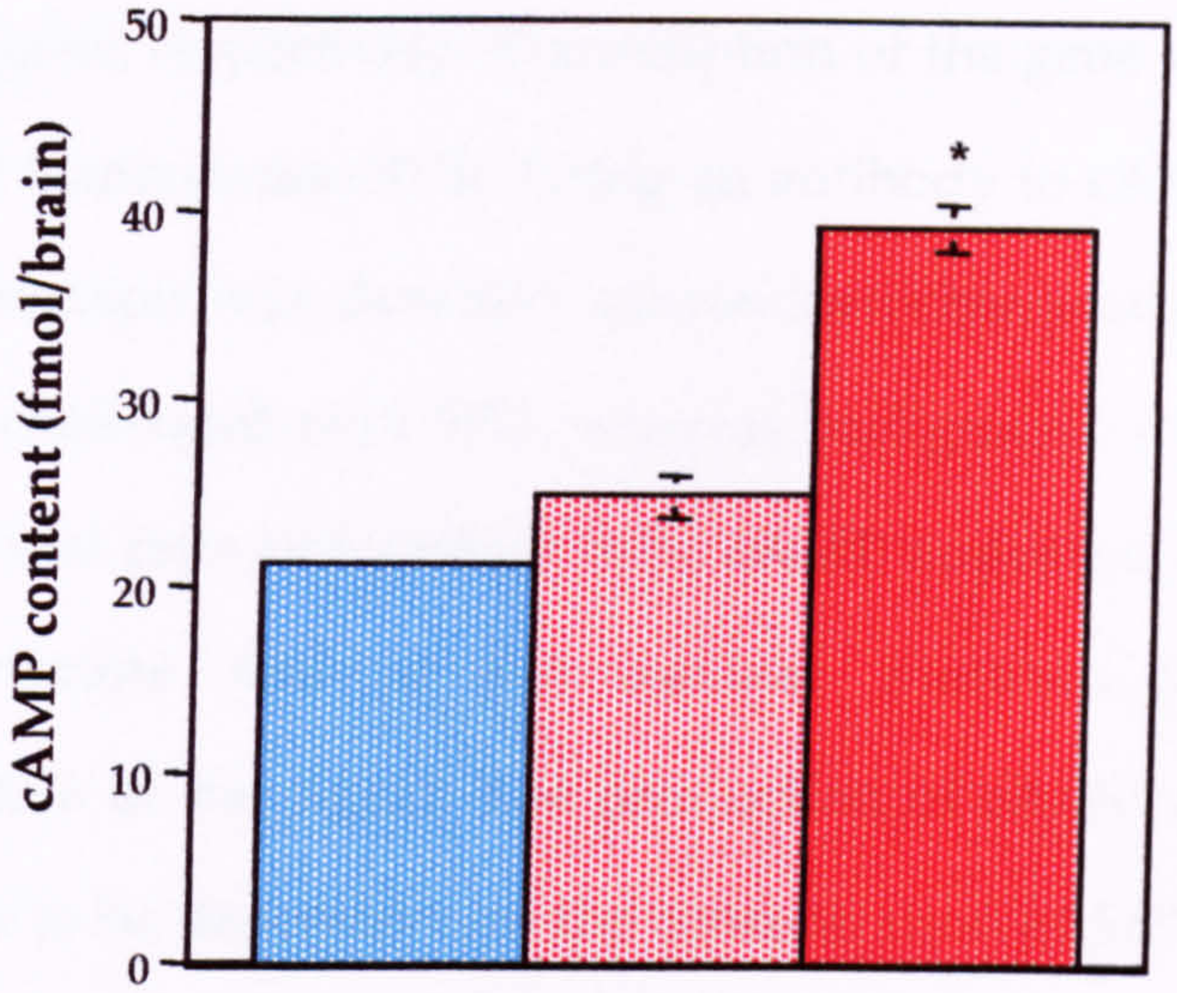
A: Ca^{2+} response to cAMP in principal cells of c42-aeq tubules.

B: Ca^{2+} response to cAMP in stellate cells of c710-aeq tubules.

4.3.13 5HT-induced elevation of cAMP in heat-shocked hs::$5HT_{7Dro}$ brains.

Flies with a P-element insertion containing $5HT_{7Dro}$ can be used to study cAMP signalling in any tissue or cell type throughout the organism. This can be achieved either by heat-shocking hs::$5HT_{7Dro}$ flies, resulting in ubiquitous expression of the gene, or by crossing UAS::$5HT_{7Dro}$ flies with any of the range of GAL4 lines that are available for targetted expression: introduction of 5HT to the fly, or a particular tissue, will stimulate the receptor to elevate intracellular cAMP levels. However, unlike ectopic expression of GC-A in flies, overexpression of $5HT_{7Dro}$ may perturb signalling in tissues that are normally affected by serotonin.

Brains (including optic lobes) were assayed for cAMP content with and without 1 μM 5HT, as in other assays, from heat-shocked and non heat-shocked hs::$5HT_{7Dro}$ flies. A small rise in cAMP content was observed in brains from heat-shocked flies, which suggests that $5HT_{7Dro}$ had been stimulated by endogenous 5HT. However, the elevation of cAMP could be greatly potentiated with 1 μM 5HT (Figure 4.16).



Heat-shock	-	+	+
5HT	+	-	+

Figure 4.16: Cyclic AMP in *Drosophila* brain is elevated by expression of $5HT_{7Dro}$ and is potentiated by treatment with 5HT

Brains were dissected from pP{CaSpeRhs::$5HT_{7Dro}$} flies (five per sample), heat-shocked and non heat-shocked. Brains were treated with the phosphodiesterase inhibitor IBMX (10^{-4} M) ± 1μM 5HT. Results are expressed as mean cAMP content (fmol/brain) ± SEM, n=3. cAMP content significantly different from basal cAMP content is denoted by *. ($P<0.05$, determined with Student's *t*-test on unpaired samples, assuming unequal variances).

4.4 Discussion

A 1.7 kb $5HT_{7Dro}$ open reading frame was subcloned into the P-element vectors pP{CaSpeRhs/act} and pP{UAST} to allow temporal and spatial control of expression of the gene, respectively. Transcription of the gene was detected in tubules by reverse transcriptase PCR. Using an antibody to cAMP, production of the second messenger was detected immunocytochemically in transgenic tubules that were challenged with 5HT, whereas background levels were very low. In both principal cells and stellate cells, cAMP appeared in pools at the basolateral membrane and at the nuclear membrane, suggesting compartmentalisation of the signal. The production of cAMP was shown by radioimmunoassay to be dependent on the concentration of 5HT used, with a mean EC_{50} of $2x10^{-8}$ M, and production could be attenuated by the 5HT receptor antagonist (+)-butaclamol with a mean IC_{50} of 2.5×10^{-8} M, consistent with previously published data (Witz *et al*, 1990; Obosi *et al*, 1996). Principal and stellate cells of the Malpighian tubules were both capable of producing cAMP at physiological ranges of 5HT. 5HT acted to elevate fluid secretion rate either when $5HT_{7Dro}$ was expressed throughout tubules, or in a cell specific manner. The maximal rates of 5HT-induced fluid secretion observed with c42/UAS::$5HT_{7Dro}$ and c710/ or c724/UAS::$5HT_{7Dro}$ tubules appear to be additive, as the sum of these rates is approximately equal to the maximal 5HT-induced rate observed in heat-shocked hs::$5HT_{7Dro}$ tubules. The specificity of cAMP as a downstream effector of $5HT_{7Dro}$ activation was determined by measuring intracellular cGMP levels in 5HT-stimulated tubules. No significant changes in intracellular cGMP levels between stimulated and unstimulated samples were observed, suggesting that $5HT_{7Dro}$ acts to specifically generate cAMP. These data show that both principal and stellate cells are capable of generating intracellular cAMP and that this leads to elevated fluid secretion rates. This is the first demonstration that Malpighian tubules of *Drosophila* can

produce cAMP in response to an extracellular ligand, and furthermore, both cell types of the main segment can do so, with physiological consequence.

$[Ca^{2+}]_i$ levels were assayed in tubules expressing $5HT_{7Dro}$ cell-specifically. Stimulation of tubules expressing $5HT_{7Dro}$ in principal cells with 5HT resulted in a biphasic elevation of $[Ca^{2+}]_i$, whereas there was no change in tubules expressing $5HT_{7Dro}$ in stellate cells. This implies that the $5HT_{7Dro}$ receptor does not directly stimulate Ca^{2+} release into the cytosol *via* activation of PLC, as if this was the mechanism of elevation of $[Ca^{2+}]_i$, it would be seen in both cell types- the lack of a calcium signal in stellate cells is not due to absence of PLC in stellate cells: the receptor for Drosokinin is a G protein-coupled receptor which causes a large PLC-induced rise in $[Ca^{2+}]_i$ in stellate cells when tubules are stimulated with the neuropeptide leucokinin (Rosay *et al*, 1997; Radford *et al*, 2002; Pollock *et al*, submitted 2002). The elevation observed in principal cells could either be due to downstream cross-talk, which does not take place in stellate cells, or that principal cells contain cyclic nucleotide gated channels that are not present in stellate cells.

Activity of cAMP-regulated phosphodiesterase(s) in $5HT_{7Dro}$ transgenic tubules was assayed, both in the presence and absence of 5HT and modulation of cAMP-regulated phosphodiesterase activity confirms a role for PDEs in control of fluid secretion. Upregulation of cAMP-dependent phosphodiesterase activity in $5HT_{7Dro}$ transgenic tubules led to concurrent increases in cAMP- and cGMP-hydrolysing phosphdiesterase activity (Figure 4.14A and B), but no significant alterations in intracellular cGMP levels were observed in 5HT-stimulated tubules (Figure 4.11). The fact that elevation of cGMP-hydrolysing activity is observed, yet cGMP levels remain unperturbed perhaps suggests that the phosphodiesterase responsible has dual substrate specificity for cAMP and cGMP, but either has a higher specificity for cAMP, or cAMP is preferentially hydrolysed simply because it is far more abundant than cGMP. The activation

of a separate cGMP-hydrolysing phosphodiesterase is, however, also plausible and could be a result of either cross-activation by cAMP, or by cross-talk further downstream of the cAMP-mediated pathway. A model for the proposed signalling events in $5HT_{7Dro}$ transgenic tubules is summarised in Figure 4.17.

RT-PCR analysis revealed the presence of transcripts for all *Drosophila* G protein subunits with the exception of Gβ76C, which strengthens the probabilty that elevated cAMP is achieved through activation of adenylate cyclase by $G\alpha_s$. A $G\alpha_i$ subunit was also detected, which implies that there are endogenous basolateral membrane receptors that elicit physiological responses through modulation of adenylate cyclase(s).

Finally, the use of $5HT_{7Dro}$ as a generic tool for manipulating cAMP in any tissue of *Drosophila* was illustrated by assaying $[cAMP]_i$ in brains taken from hs::$5HT_{7Dro}$ flies, both heat-shocked and non heat-shocked in the presence and absence of 5HT. The potentiated elevation of $[cAMP]_i$ in brains stimulated with 5HT demonstrates that this work could be extended to studying the effects on learning and memory and circadian rhythms; an exciting prospect, as cAMP is already known to have an important role in learning and memory on the basis of learning deficits seen in flies with mutations in genes in the cAMP-signalling cascade (*dunce* and *rutabaga*) (Dudai *et al*, 1976; Livingstone *et al*, 1984; Chen *et al*, 1986; Levin *et al*, 1992). While some brain cells clearly already express endogenous $5HT_{7Dro}$, the large elevation of intracellular cAMP content in hs::$5HT_{7Dro}$ brains, in response to 5HT (Figure 4.16), suggests that most neurons are only responsive in transgenic flies, and so a specific GAL4 driver directing $5HT_{7Dro}$ to particular cell types may be a very useful tool.

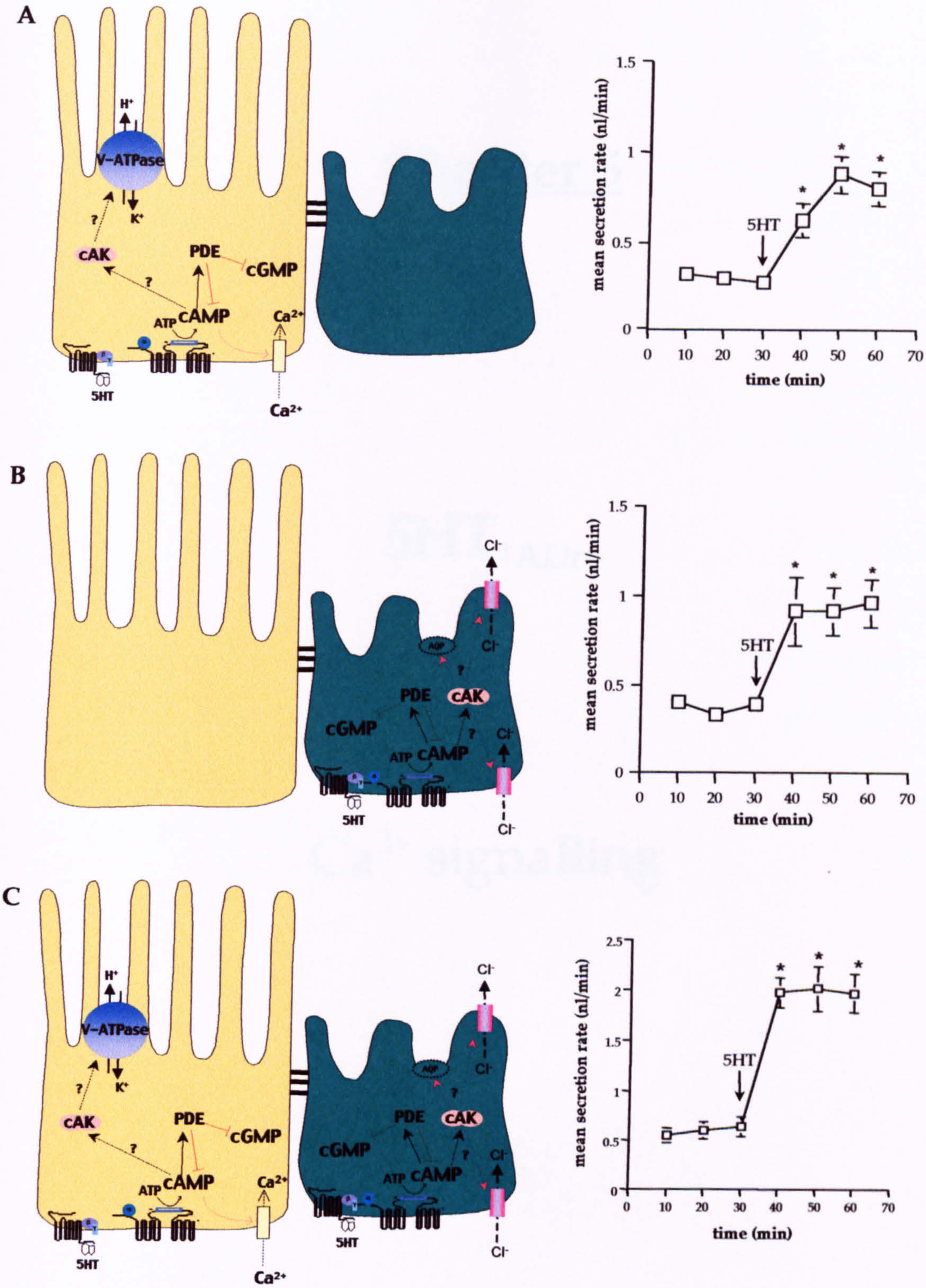


Figure 4.17: A model for cell-specific action of cAMP in Malpighian tubules

A: 5HT-induced cell-specific generation of cAMP in principal cells elevates fluid secretion *via* cAMP-dependent kinase (cAK), and elevation of $[Ca^{2+}]_i$, to stimulate V-ATPase activity.

B: 5HT-induced cell-specific generation of cAMP in stellate cells elevates fluid secretion *via* cAMP-dependent kinase (cAK), to increase chloride conductance.

C: 5HT-induced ubiquitous generation of cAMP in Malpighian tubules has an additive effect on fluid secretion.

Chapter 5

$5HT_{1ADro}$

&

Ca^{2+} signalling

5.1 Summary

Transgenic flies containing the *Drosophila* serotonin receptor gene $5HT_{1ADro}$ under UAS control were a gift from Frédéric Saudou, University L. Pasteur de Strasbourg. This receptor has previously been shown to both increase inositol phosphate production and inhibit forskolin-stimulated elevation of cAMP in mammalian cell culture. Expression of $5HT_{1ADro}$ in Malpighian tubules confers sensitivity to 5HT in a dose-dependent manner with an EC_{50} of approximately 5 x 10^{-8}M, determined by fluid secretion assay. However, in the presence of the $5HT_{1ADro}$ antagonist, yohimbine, elevation of fluid secretion is attenuated, but not completely inhibited.

The activation of $5HT_{1ADro}$ with 5HT was shown to elevate $[Ca^{2+}]_i$ in both principal and stellate cells of tubules by aequorin bioluminesence assay. However, the different cell types had different profiles of calcium response, indicating that the cells were responding in a different manner from one another. Interestingly, these calcium responses did not appear to influence intracellular levels of the second messengers cAMP and cGMP.

5.2 Introduction

Many cellular functions are controlled by modulation of the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), including gene expression, contraction, metabolism and secretion. Regulation of numerous functions within the same cell requires that both Ca^{2+} delivery can span the whole cell and that intracellular concentrations of Ca^{2+} are tightly controlled. Modulation of $[Ca^{2+}]_i$ cannot generally be controlled simply by the influx of Ca^{2+} because of its low diffusibility in the cytoplasm. Therefore, the endoplasmic reticulum (ER) acts as a Ca^{2+} store to deliver Ca^{2+} in such a way as to allow compartmentalisation and site-specific delivery without compromising cell homeostasis. In fact, high $[Ca^{2+}]_i$ over prolonged periods of time can be damaging to cells, and because of this cytotoxicity $[Ca^{2+}]_i$ in resting cells is normally held between 20-100 nM. A Ca^{2+}-ATPase continually pumps Ca^{2+} from the cytoplasm back into the ER to maintain this low intracellular concentration (Berridge, 1997).

As described earlier, Ca^{2+} has distinct roles in specific cell types of *Drosophila* Malpighian tubules. Members of the leucokinin family of neuropeptides elevate fluid secretion through stimulation of a G protein-coupled receptor, that acts to raise $[Ca^{2+}]_i$ in stellate cells (O'Donnell *et al*, 1996; Rosay *et al*, 1997; Radford *et al*, 2002). This action has been shown to raise chloride shunt conductance, which can be blocked with the calcium chelator BAPTA-AM (O'Donnell *et al*, 1996). Current density hot spots, coincident with stellate cells, were revealed with self-referencing probe analysis. These hot spots could be abolished with chloride channel blockers or low-chloride saline, suggesting that chloride conductance is confined to stellate cells (O'Donnell *et al*, 1997). Recently, the receptor for Drosokinin, named DLKR, has been localised to stellate cells of Malpighian tubules, which further supports the model for a stellate cell-specific Ca^{2+} signal, that elevates stellate cell-specific chloride conductance (Radford *et al*, 2002). A principal cell-specific Ca^{2+} signal is also necessary for CAP_{2b}/capa-induced

elevation of fluid secretion (Rosay *et al*, 1997; Kean *et al*, 2002). This Ca^{2+} signal is necessary to activate a Ca^{2+}-dependent NOS, which stimulates production of NO. This, in turn, stimulates a soluble guanylate cyclase to produce cGMP, with elevation of fluid secretion being the end result (Dow *et al*, 1994a; Davies *et al*, 1995; Davies *et al*, 1997). However, elevated $[cGMP]_i$ has been shown to induce a $[Ca^{2+}]_i$ rise in principal cells through a process dependent on Ca^{2+} entry, and can be blocked with calcium channel antagonists (MacPherson *et al*, 2001). The action of cyclic nucleotides in principal cells involves stimulation of an apical V-ATPase, which drives a proton/alkali metal ion exchanger (antiporter), determined by the increase in lumen electropositive potential in response cAMP or cGMP (O'Donnell *et al*, 1997). It has been demonstrated in the previous chapters that elevation of cyclic nucleotides induce Ca^{2+} signals in principal cells, but not in stellate cells. This implicates Ca^{2+} as an important modulator of principal cell function, which is distinguishable from its role in stellate cells. It is therefore desirable to manipulate Ca^{2+} in a cell-specific manner, using the same technique deployed to manipulate cGMP and cAMP in the previous chapters. Using a receptor that is known to stimulate $[Ca^{2+}]_i$ in other systems, it would be possible to elevate Ca^{2+}, not just in principal and stellate cells of the main segment, but also in regions of the tubule (such as the initial segment) that are involved in Ca^{2+} transport.

Transgenic flies containing the *Drosophila* serotonin receptor gene $5HT_{1ADro}$ under UAS control were a gift from Frédéric Saudou, University of Strasbourg. This is a G protein-coupled receptor that has been shown to elevate intracellular $InsP_3$ in response to 5HT (Saudou *et al*, 1992). Expression of this transgene under UAS control allows both study the pharmacology of this receptor *in vivo* to be studied and the Ca^{2+} signal transduction pathway to be isolated. This enables cross-talk with the other second messenger pathways discussed previously to be examined. As in the previous chapter, serotonin, the ligand for

this receptor, does not normally elicit a response in isolated Malpighian tubules, making this receptor a good choice as a modulator of $[Ca^{2+}]_i$.

The $5HT_{1ADro}$ receptor was cloned by Saudou *et al* (1992) and designated 5HT-dro2A. Heterologous expression studies of this receptor in mammalian cells demonstrated that PLC was activated with concurrent inhibition of adenylate cyclase when stimulated with 5HT (Saudou *et al*, 1992). The 5HT-dro2A receptor induced a decrease in the levels of cAMP stimulated by forskolin and induced a 1.5- to 2-fold increase in the level of inositol phosphates in response to 5HT (Table 1.2). Furthermore, pertussis toxin partially blocked the effect of this receptor, indicating that the receptor interacts with a pertussis toxin-sensitive G protein. This evidence suggests that Ca^{2+} plays a key role in the signal transduction of this receptor, and targetted expression of this receptor is therefore potentially useful for targetted manipulation of Ca^{2+}. As with $5HT_{7Dro}$, discussed in the previous chapter, this receptor requires its cognate G protein subunits to be expressed in the particular cell to which it is targetted. There is good evidence that this is the case: both the receptors for CAP_{2b} and for Drosokinin are GPCRs that modulate $[Ca^{2+}]_i$, and there is only one G_q gene in the *Drosophia* genome, which has been shown to be expressed in tubules by RT-PCR (Figure 4.14).

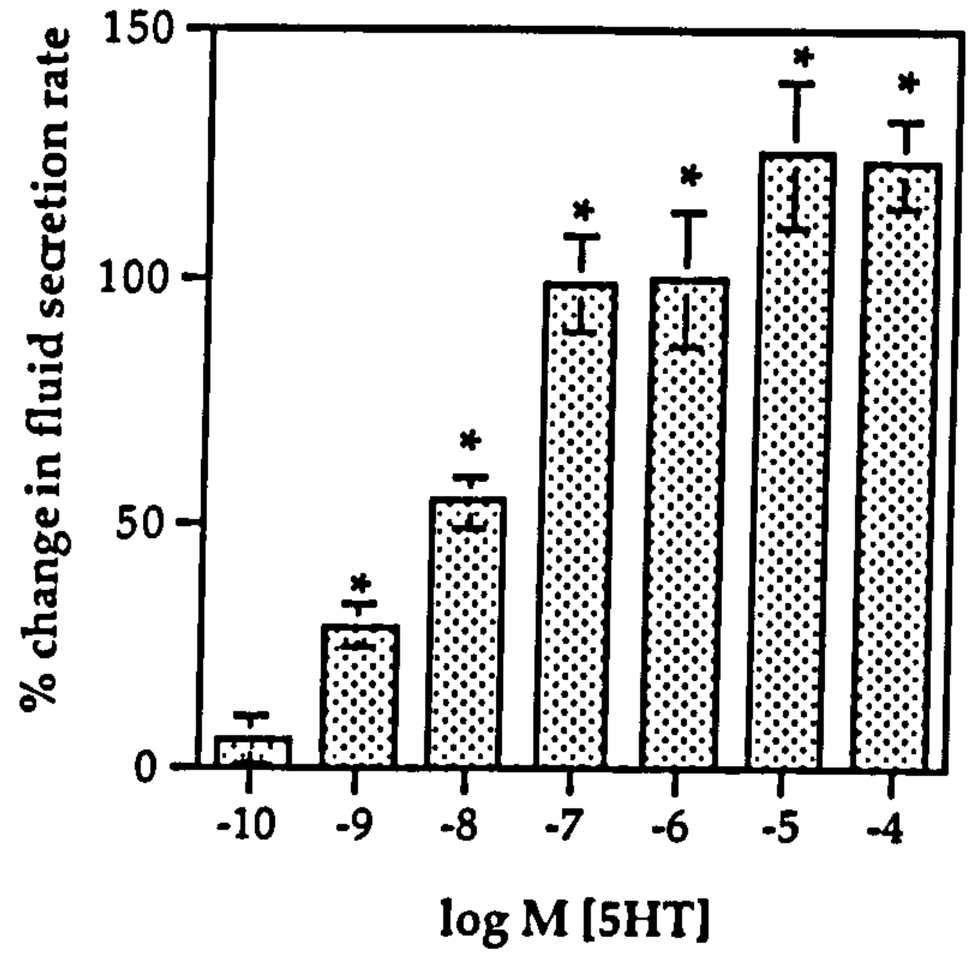
It is now apparent, that of mammalian 5HT receptors, 5HT-dro2A shares greatest similarity and transduction properties with the human $5HT_{1A}$ receptor and is regarded as an invertebrate $5HT_1$-like receptor (Gerhardt *et al*, 1996). It has therefore been renamed $5HT_{1ADro}$ (and similarly 5HT-dro2B, also cloned by Saudou *et al*, renamed $5HT_{1BDro}$) in an attempt to standardize nomenclature of this large family of receptors (Colas *et al*, 1997; Tierney 2001; Table 1.1).

5.3 Results

5.3.1 Targetted expression of $5HT_{1ADro}$ in tubules confers 5HT-induced elevation of fluid transport.

Progeny of a cross between the strain pP{UAS::$5HT_{1ADro}$} and GAL4 line c42 (referred to as c42/UAS::$5HT_{1ADro}$) would be expected to express $5HT_{1ADro}$ in principal cells of the main segment of tubules. Similarly, progeny of a cross between the strain pP{UAS::$5HT_{1ADro}$} and GAL4 lines c724 or c710 (referred to as c724/UAS::$5HT_{1ADro}$ and c710/UAS::$5HT_{1ADro}$ respectively) could be expected to express $5HT_{1ADro}$ in stellate cells of tubules. Tubules were dissected for fluid secretion assays and fluid secretion rate was measured for 30 min before 5HT was added to the bathing solution. The fluid secretion rate was measured for a further 30 min. Calculations of fluid secretion rate were made as described in Chapter 3. All tubules expressing $5HT_{1ADro}$ displayed sensitivity to serotonin (5HT) in a dose-dependent manner. Figure 5.1A shows the dose response of c42/UAS::$5HT_{1ADro}$ tubules to 5HT, expressed as a percentage rise above basal secretion rate. Peak rates of fluid secretion were observed at 10 μM 5HT and half-maximal stimulation was achieved between 10^{-8} M and 10^{-7} M, consistent with the EC_{50} ($2x10^{-8}$M) for the action of this receptor to decrease forskolin-stimulated cAMP production in mammalian cells (Saudou *et al*, 1992). A dose response curve was similarly generated using c724/UAS::$5HT_{1ADro}$ tubules (Figure 5.1B). As with expression of this receptor in principal cells, maximal elevation of fluid secretion was achieved with 1-100 μM 5HT and half-maximal elevation of fluid secretion was achieved with between 10^{-8} M and 10^{-7} M 5HT. In all tubules expressing the $5HT_{1ADro}$ receptor, there was a significant elevation of fluid secretion rate above basal with concentration of 5HT as low as 10^{-9} M, which was sustained beyond 30 min post-stimulation. These results indicate that the receptor behaves as expected from previous work in cell lines (Saudou *et al*, 1992).

A



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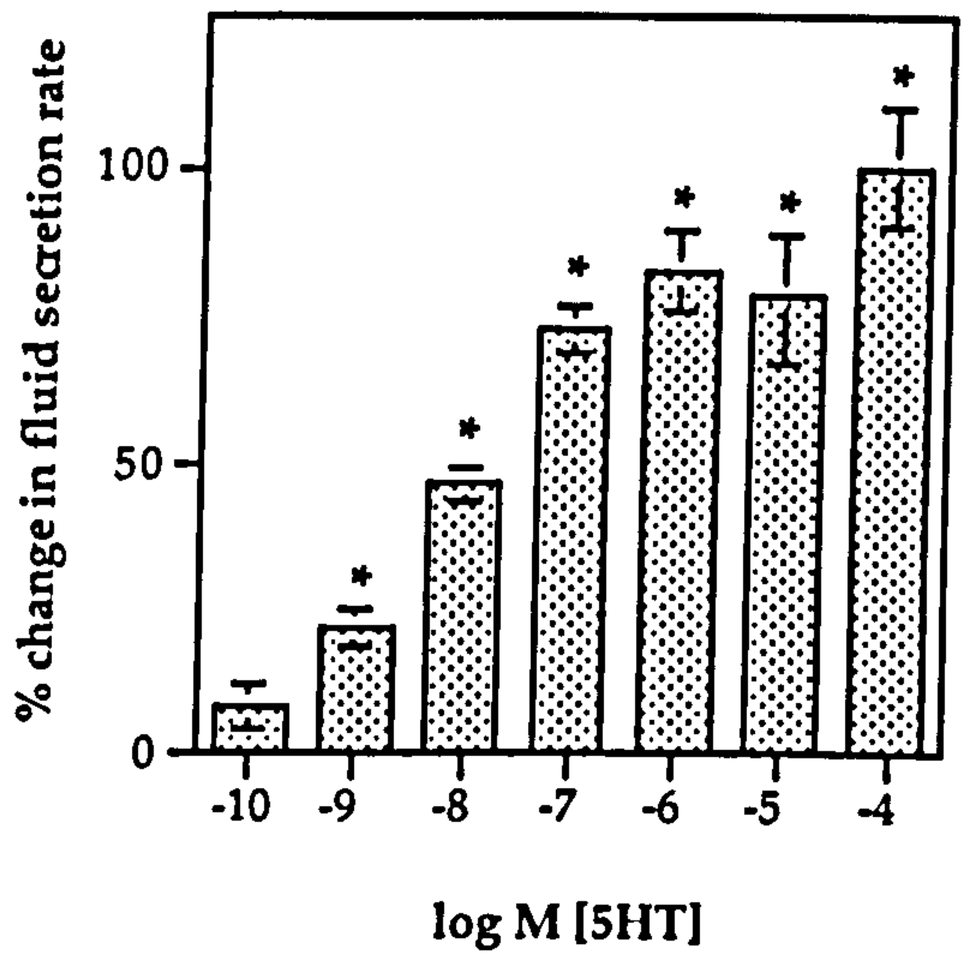


Figure 5.1: Fluid secretion is elevated by 5HT in GAL4/UAS::5HT$_{1ADro}$ tubules in a dose dependent manner

Tubules from GAL4/UAS:: 5HT$_{1ADro}$ were challenged with 5HT at 30 min.
A: c42/UAS::5HT$_{1ADro}$ tubules challenged with 5HT showed elevated fluid secretion rates over basal controls. Results are expressed as a percentage change from basal secretion rates ± SEM, n=10.
B: c724/UAS::5HT$_{1ADro}$ tubules challenged with 5HT showed elevated fluid secretion rates over basal controls. Results are expressed as a percentage change from basal secretion rates ± SEM, n=10.

Secretion rates significantly different from basal are denoted by *. ($P<0.05$, determined with the Student's *t*-test, on unpaired samples, assuming unequal variances).

5.3.2 Yohimbine inhibits 5HT-induced elevation of fluid secretion in $5HT_{1ADro}$ tubules

Yohimbine is known to be a selective inhibitor of 5HT receptors (see Table 1.2), with a K_i of 18 μM for the $5HT_{1ADro}$ receptor (Saudou *et al*, 1992). Therefore, if the transgenic receptor is acting physiologically in Malpighian tubules, the action of 5HT should be inhibited by yohimbine.

Figure 5.2A shows a typical fluid secretion assay on c42/UAS::$5HT_{1ADro}$ flies. The response to 5HT can be attenuated when tubules are pretreated with 10 fold excess of the $5HT_{1ADro}$ antagonist yohimbine.

Figure 5.2B shows a typical fluid secretion assay on c724/UAS::$5HT_{1ADro}$ flies. Similarly, the response to 5HT can be attenuated when tubules are pretreated with 10 fold excess of the $5HT_{1A}$ antagonist yohimbine.

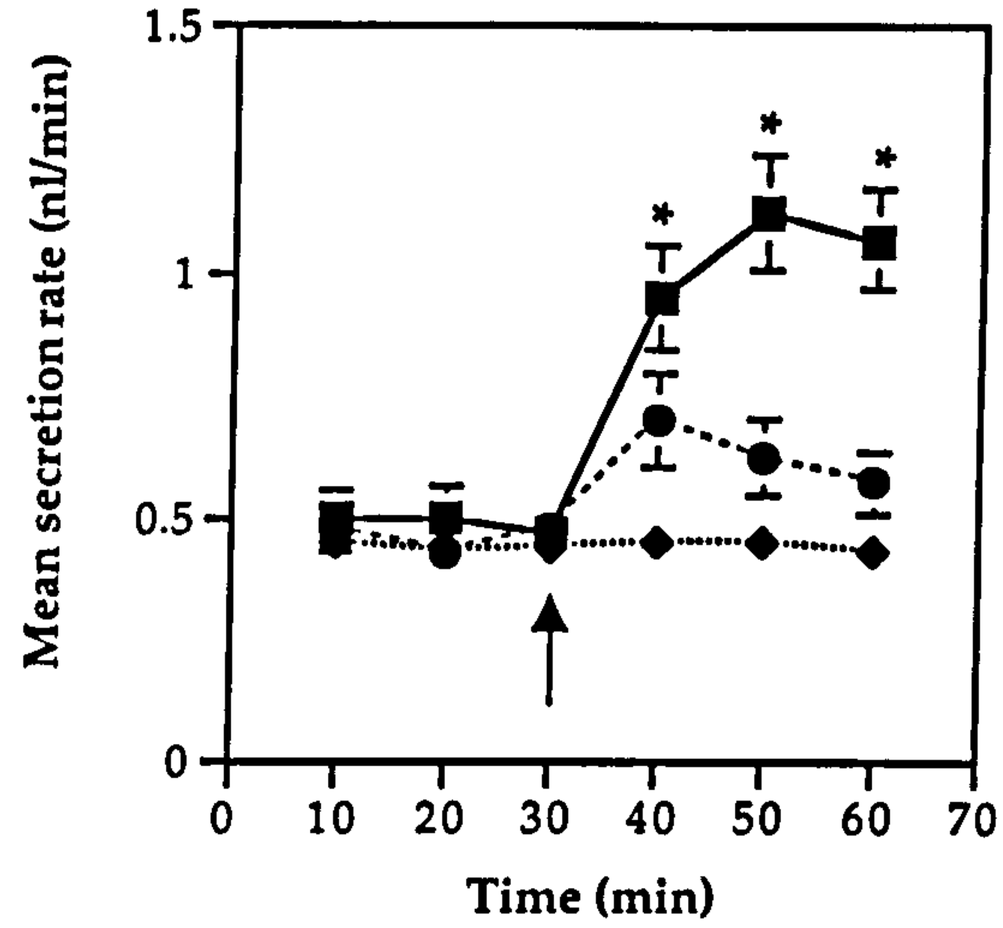
5.3.3 5HT-stimulated tubules expressing the $5HT_{1ADro}$ transgene elevate intracellular Ca^{2+} levels.

As activation of $5HT_{1ADro}$ in mammalian cell culture has been shown to increase inositol phosphate production, it would therefore be expected that this would result in Ca^{2+} release from internal stores, through $InsP_3$ receptors. Flies with $5HT_{1ADro}$ under control of UAS were crossed with flies expressing both GAL4 and aequorin cell-specifically (c42;aeq and c710;aeq) to determine the effect of receptor activation on Ca^{2+} release. Indeed, 5HT-stimulated tubules expressing $5HT_{1ADro}$ in principal cells responded with a sharp initial rise in $[Ca^{2+}]_i$ which only lasted approximately 20-30 s (peaking at 160 nM), followed by a smaller, more sustained rise, lasting 1-2 min (peaking at 110 nM). However, the $[Ca^{2+}]_i$ did not return to basal levels for 10-15 min post-stimulation (Figure 5.3A) This response does not mirror that of the CAP_{2b}-induced Ca^{2+} rise, which has been shown to involve a principal cell-specific rise in $[Ca^{2+}]_i$ response (Rosay *et al*, 1997; Kean *et al*, 2002; MacPherson *et al*, submitted 2002). The amplitude of the

initial Ca^{2+} peak in c42/UAS::$5HT_{1ADro}$ tubules is lower than that of tubules stimulated with CAP_{2b}. Furthermore, the secondary rise in c42/UAS::$5HT_{1ADro}$ tubules has a different profile: almost 1 min elapses before the rise begins, which, again is lower in amplitude than that of the CAP_{2b}-induced secondary response. The Ca^{2+} response seen in c710/UAS::$5HT_{1ADro}$ tubules to 5HT (Figure 5.3B) is also somewhat different from Ca^{2+} traces observed in tubules stimulated with the endogenous neuropeptide, Drosokinin. The calcium signal generated from stimulation with Drosokinin has been shown to be stellate-cell specific (Rosay *et al*, 1997; Terhzaz *et al*, 1999; Radford *et al*, 2002) and a G protein-coupled receptor has been identified as its receptor (Radford *et al*, 2002). The response to Drosokinin is seen as a large initial rise in $[Ca^{2+}]_i$, followed by an immediate secondary rise, resembling two peaks within the space of approximately 20 s. However, when tubules express the $5HT_{1ADro}$ in stellate cells, the calcium response to 5HT is seen as an initial rise in $[Ca^{2+}]_i$ which peaks at 120 nM and lasts approximately 40-50 s, before a smaller secondary rise in $[Ca^{2+}]_i$ lasting for more than 5 min (Figure 5.3B). A prolonged secondary rise in stellate cell $[Ca^{2+}]_i$ has not previously been described, suggesting that the calcium signal generated by activation of this receptor is not the same as the signal generated by the leucokinin receptor.

Interestingly, when the $5HT_{1ADro}$ receptor was expressed ubiquitously throughout tubules by heat-shock (from a cross between UAS::$5HT_{1ADro}$ and aeq;hsGAL4 flies), the initial calcium peak reaches 350 nM, which is substantially more than the sum of the c42/$5HT_{1ADro}$ and c710/$5HT_{1ADro}$ initial peaks. (Figure 5.4) The initial rise is of approximately the same duration as the c710/$5HT_{1ADro}$ peak, followed by the secondary peak observed in c42/$5HT_{1ADro}$ tubules (peaking at 125 nM; slightly larger than the c42/$5HT_{1ADro}$ peak). There is also a sustained elevation of $[Ca^{2+}]_i$ which was seen in both c42/$5HT_{1ADro}$ and c710/$5HT_{1ADro}$ for several minutes.

A



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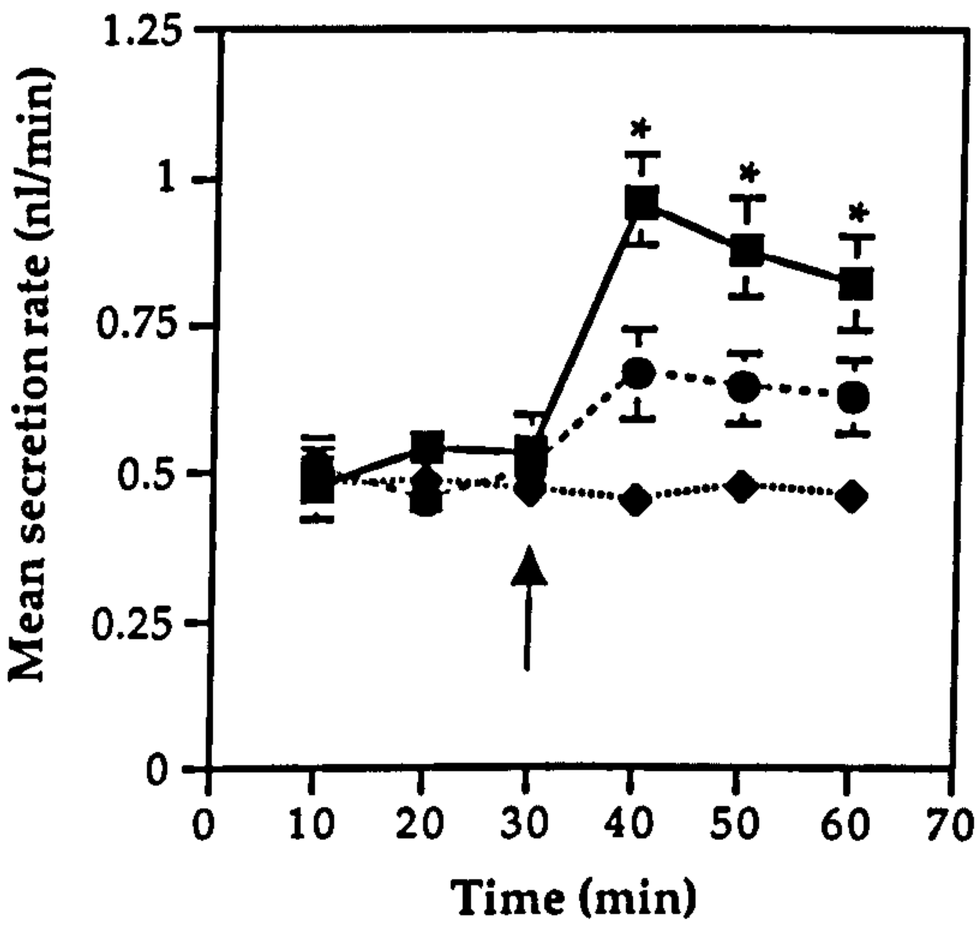

Figure 5.2: 5HT-induced stimulation of fluid secretion in tubules expressing $5HT_{1ADro}$ can be inhibited by the $5HT_{1ADro}$ antagonist yohimbine

A: Tubules expressing $5HT_{1ADro}$ in principal cells (c42/UAS::$5HT_{1ADro}$) challenged with 1 μM 5HT at 30 min (arrow) showed elevated fluid secretion rates (■) over non-treated tubules (◆). Tubules treated with the $5HT_{1ADro}$ antagonist yohimbine (10^{-5} M) at t=0 showed an attenuated response to 5HT (●). Results are expressed as mean fluid secretion rate (nl/min) ± SEM, n=10.
B: Tubules expressing $5HT_{1ADro}$ in stellate cells (c724/UAS::$5HT_{1ADro}$) challenged with 1 μM 5HT at 30 min (arrow) showed elevated fluid secretion rates (■) over non-treated tubules (◆). Tubules treated with the $5HT_{1ADro}$ antagonist yohimbine (10^{-5} M) at t=0 showed an attenuated response to 5HT (●). Results are expressed as mean fluid secretion rate (nl/min) ± SEM, n=10.

Secretion rates significantly different from basal are denoted by *. ($P<0.05$, determined with the Student's *t*-test, on unpaired samples, assuming unequal variances).

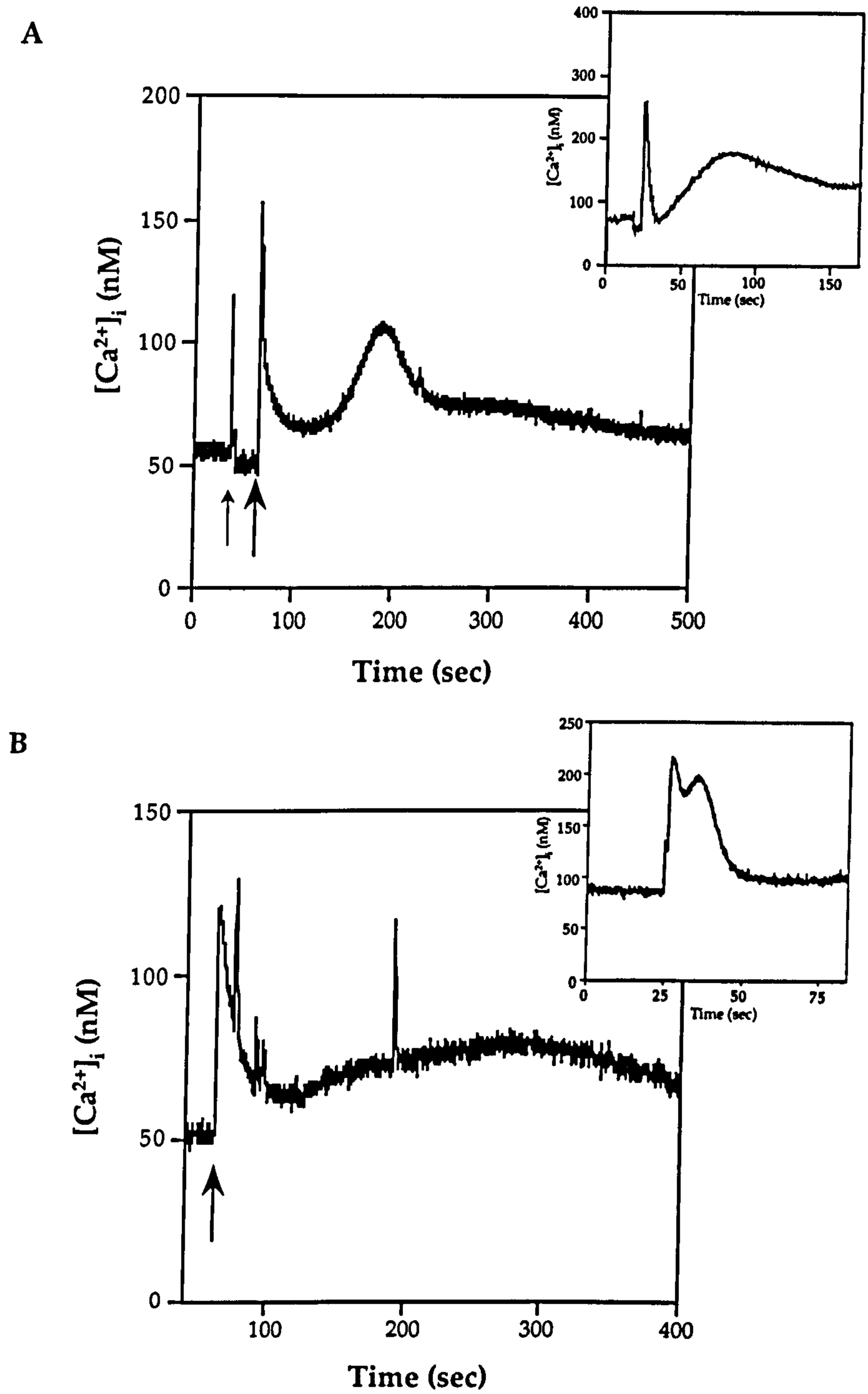

Figure 5.3: 5HT-induced $[Ca^{2+}]_i$ rises in tubules expressing $5HT_{1ADro}$ cell-specifically: representative traces

Typical traces of changes in $[Ca^{2+}]_i$ in tubule cells expressing $5HT_{1ADro}$ when stimulated with $1x10^{-8}$ M 5HT (large arrow).

A: aeq; +/+; c42 GAL4/$5HT_{1ADro}$. Inset: typical Ca^{2+} trace of CAP_{2b}-induced Ca^{2+} rise in principal cells (taken from MacPherson *et al*, submitted 2002).

B: aeq; +/+; c710 GAL4/$5HT_{1ADro}$. Inset: typical Ca^{2+} trace of Drosokinin-induced Ca^{2+} rise in stellate cells (taken from Radford *et al*, 2002).

Peaks prior to the main rise in $[Ca^{2+}]_i$ are caused by control ("mock") injections of Schneider's medium (small arrow).

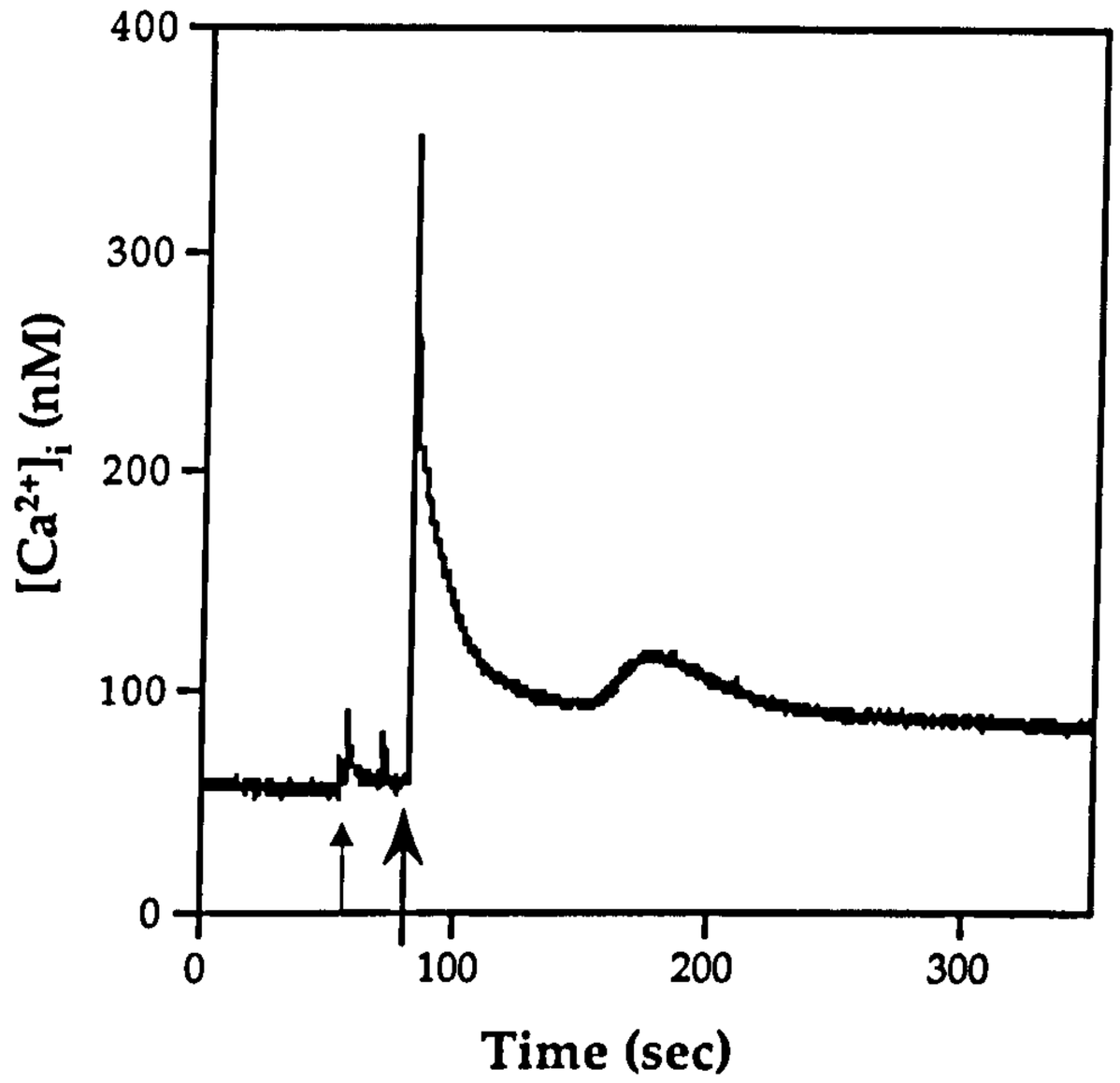

Figure 5.4: 5HT-induced $[Ca^{2+}]_i$ rises in tubules expressing $5HT_{1ADro}$ ubiquitously: representative trace

Typical trace of changes in $[Ca^{2+}]_i$ in tubule cells expressing $5HT_{1ADro}$ ubiquitously (fly line: aeq; hsGAL4/+;$5HT_{1ADro}$/+) when stimulated with $1x10^{-8}$ M 5HT (large arrow).

The peak prior to the main rise in $[Ca^{2+}]_i$ is caused by a control ("mock") injection of Schneider's medium (small arrow).

5.3.4 5HT does not alter cyclic nucleotide levels in $5HT_{1ADro}$ transgenic tubules

The possibility that intracellular cGMP levels may be altered as a result of cross-talk upon activation of $5HT_{1ADro}$ was investigated by assaying levels both with and without 5HT stimulation in samples from tubules expressing $5HT_{1ADro}$ and from a wild type control (Figure 5.5). All samples contained approximately the same basal levels of intracellular cGMP, and there was no significant change in these levels upon 5HT-induced $5HT_{1ADro}$ activation.

Similarly, cAMP levels in c42/UAS::$5HT_{1ADro}$ and c710/UAS::$5HT_{1ADro}$ tubules were assayed with and without stimulation of $5HT_{1ADro}$ with 5HT (Figure 5.6). Again, there was no significant alteration of cAMP levels in the presence of 5HT, suggesting that this calcium signal does not contribute to a cAMP-generating pathway. However, Saudou *et al* (1992) reported that forskolin-activated adenylate cyclase activity was inhibited by $5HT_{1ADro}$ activation. No decrease in basal levels of cAMP were observed in 5HT-stimulated tubules. This leads to one of two possibilities: the $5HT_{1ADro}$ receptor couples with a G protein that leads to PLC activation, but not to adenylate cyclase inhibition in *Drosophila* epithelial cells; or more simply, under these conditions there is very little adenylate cyclase activity, and inhibition of this enzyme can only be unmasked when it is activated by forskolin.

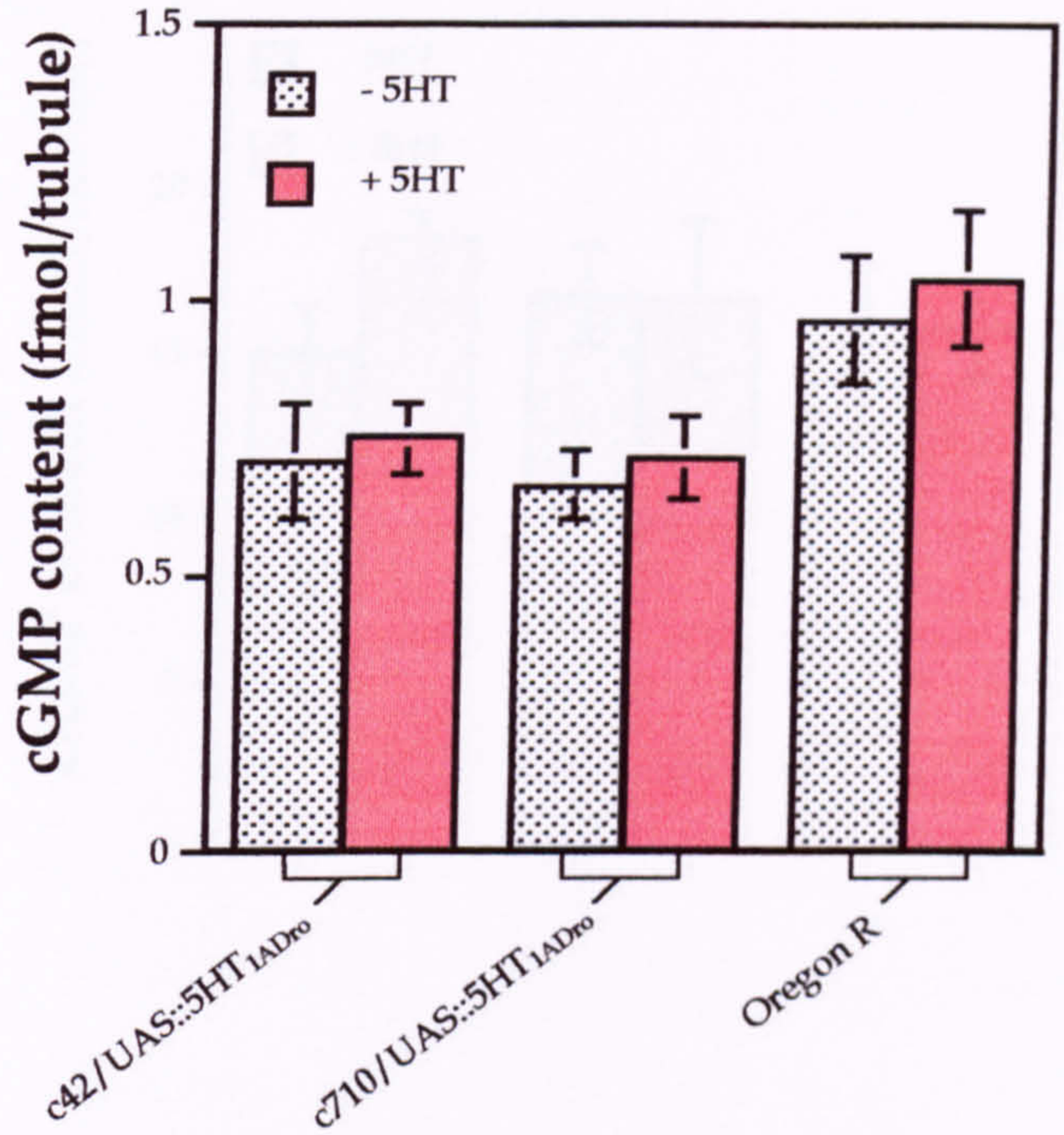

Figure 5.5: $5HT_{1ADro}$ transgenic tubules do not have significantly altered cGMP levels in response to 5HT

Tubules were treated with 5HT (1 μM) and Zaprinast (1 μM) for 10 min and prepared as described in Materials and Methods. Results are expressed as mean cGMP content (fmol/tubule) ± SEM, n=3. Differences between 5HT-treated samples and control samples are not significant. ($P>0.05$, determined with Student's *t*-test on unpaired samples, assuming unequal variances).

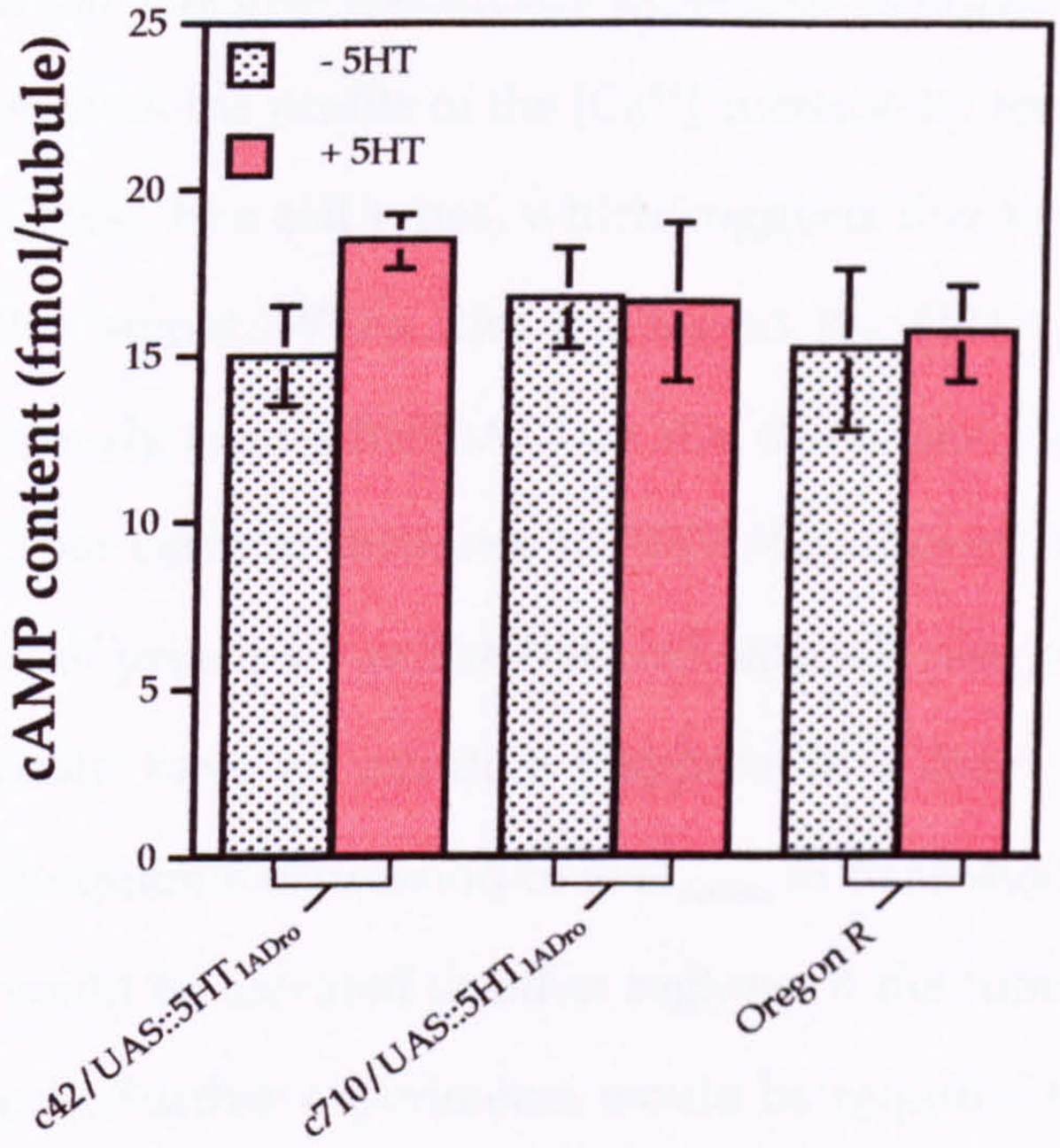

Figure 5.6: $5HT_{1ADro}$ transgenic tubules do not have significantly altered cAMP levels in response to 5HT

Tubules were treated with 5HT (1 μM) and IBMX (10^{-4} M) for 10 min and prepared as described in Materials and Methods. Results are expressed as mean cAMP content (fmol/tubule) ± SEM, n≥3. Differences between 5HT-treated samples and control samples are not significant. ($P>0.05$, determined with Student's *t*-test on unpaired samples, assuming unequal variances).

5.4 Discussion

Flies expressing $5HT_{1ADro}$ in a cell-specific manner responded to 5HT in a dose dependent manner in fluid secretion assays. The pharmacological profile of this receptor was similar whether specifically expressed in either principal cells or stellate cells. However, the profile of the $[Ca^{2+}]_i$ increase by receptor stimulation was different in these two cell types, which suggests that there is differential processing of this signal. When flies expressed the $5HT_{1ADro}$ receptor and aequorin ubiquitously (using hs::GAL4 as the driver for UAS::$5HT_{1ADro}$ and UAS::aequorin), the calcium response to 5HT stimulation was considerably larger than those of principal- and stellate cell-specific assays, indicating that these signals would have an additive response in a fluid secretion assay. However, with ubiquitous expression of $5HT_{1ADro}$ in heat-shocked hs::$5HT_{1ADro}$ tubules, $[Ca^{2+}]_i$ would be elevated in other regions of the tubule (for example, the initial segment). Further experiments would be required to determine the effects of ubiquitous Ca^{2+} modulation on fluid secretion.

No variation in intracellular cGMP levels resulted from stimulation of $5HT_{1ADro}$ tubules with 5HT, suggesting that there is not cross-talk between these two signalling pathways, when $[Ca^{2+}]_i$ levels are elevated in tubules by this method. This is surprising, as it has been demonstrated that tubules treated with the neuropeptide CAP_{2b} display an initial rise in $[Ca^{2+}]_i$ in principal cells, which ultimately leads to generation of cGMP by a soluble guanylate cyclase. Indeed a Ca^{2+} signal, and Ca^{2+} entry, has been shown to be essential in CAP_{2b} signalling (Rosay *et al*, 1997). However, the calcium signal generated by activation of $5HT_{1ADro}$ in principal cells is slightly different from that induced by CAP_{2b}: the initial 5HT-induced rise in $[Ca^{2+}]_i$ in c42/UAS::$5HT_{1ADro}$ tubules is smaller than the CAP_{2b}-induced initial $[Ca^{2+}]_i$ rise observed in c42;aeq tubules. The secondary rises in $[Ca^{2+}]_i$ are also different: although both responses involve prolonged elevation of $[Ca^{2+}]_i$ over several minutes, the 5HT-induced secondary response

only occurs approximately 1 min after the initial $[Ca^{2+}]_i$ rise, whereas the CAP_{2b}-induced $[Ca^{2+}]_i$ rise follows immediately after the initial rise. These results may outline the importance of spatio-temporal Ca^{2+} signalling in Malpighian tubules. A further possibility is that there is an association of a regulatory protein(s) to the receptor for CAP_{2b}, which does not associate with the $5HT_{1ADro}$ receptor, meaning the Ca^{2+} signal is differentially recognised.

Interestingly, there was no reduction in basal levels of cAMP in tubules expressing $5HT_{1ADro}$ when stimulated with 5HT. However, the work by Saudou *et al* demonstrated that forskolin-stimulated cAMP levels were reduced when cells expressing $5HT_{1ADro}$ were stimulated with 5HT, concluding that the G protein that associates with $5HT_{1ADro}$ inhibited adenylate cyclase activity. This leads to one of two possibilities: the $5HT_{1ADro}$ receptor couples with a G protein that leads to PLC activation, but not to adenylate cyclase inhibition in *Drosophila* epithelial cells; or more simply, under these conditions there is very little adenylate cyclase activity, and inhibition of this enzyme can only be unmasked when it is activated by forskolin. A similar experiment to that of Saudou *et al* would need to be performed to determine which of these possibilities is correct. A model for the Ca^{2+} signalling mechanisms induced by stimulation of the $5HT_{1ADro}$ is illustrated in Figure 5.7.

Ca^{2+} signalling is extremely important, and expression of the $5HT_{1ADro}$ transgene can potentially be utilised to modulate PLC-activated Ca^{2+} release in any cell-type, with only tissue sensitivity to 5HT being a limitation. In the context of Malpighian tubules, cell-specific $5HT_{1ADro}$ expression may prove to be useful to study flies that are defective in some component of Ca^{2+} signalling, for example $InsP_3$ receptor mutants.

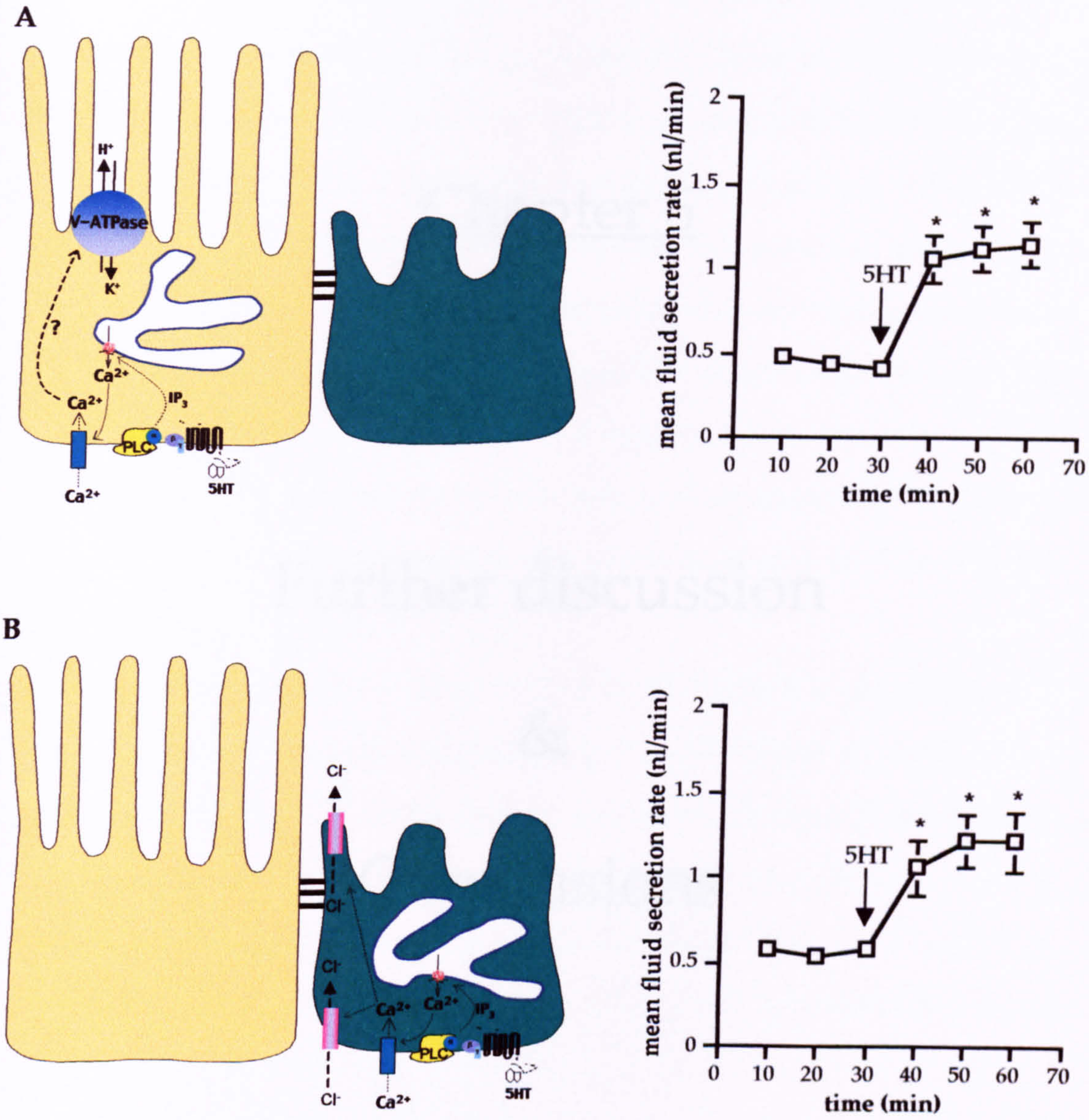

Figure 5.7: A model for cell-specific action of Ca^{2+} in $5HT_{1ADro}$ transgenic Malpighian tubules

A: 5HT-induced principal cell-specific generation of $InsP_3$ induces Ca^{2+} release from the ER. This, in turn, stimulates Ca^{2+} entry which elevates fluid secretion, possibly by stimulation of V-ATPase activity.

B: 5HT-induced stellate cell-specific generation of $InsP_3$ induces Ca^{2+} release from the ER. This, in turn, stimulates Ca^{2+} entry which elevates fluid secretion, by increasing chloride shunt conductance.

Chapter 6

Further discussion

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Conclusions

6.1 Summary

This work has aimed to further our knowledge of how the Malpighian tubule of *Drosophila* integrates cell-specific signal cascades to result in overall control and modulation of function. Furthermore, it has established that *Drosophila* is a good model organism for *in vivo* heterologous receptor pharmacology, which has the advantage over cell culture in that receptors are expressed in a differentiated, multicellular environment. This allows subtleties to be detected that may not be seen in the bland, de-differentiated background of cell lines. Obviously, the closer the cellular context from which a receptor has been taken, the more relevant the information about that receptor becomes, and if the cells themselves have not been removed from their natural context, there is further enhancement of relevance.

As discussed at the end of chapters 3, 4 and 5, there is a great amount of complexity to the signal transduction pathways known to control fluid transport in Malpighian tubules. Even for such a morphologically simple tissue, the cross-talk, cross-activation and overall integration of signals is not easy to unravel. This work has built a platform for further dissection of signalling events by allowing activation of cGMP, cAMP and Ca^{2+} to be isolated from one another.

Many of the signalling events which contribute to fluid transport of the tubule have previously been characterised (Davies *et al*, 1995; Davies *et al*, 1997; Rosay *et al*, 1997; Davies, 2000; MacPherson *et al*, 2001). This was discussed in chapter 1 and is summarised in Figure 1.6. We now have a better understanding of what effectors and negative regulators are modulated in response to second messenger stimulation, summarised in Figure 6.1. A brief recapitulation of results from this work with additional comments is given below.

6.1.1 GC-A and cGMP signalling.

Transgenic tubules expressed the rat guanylate cyclase receptor GC-A on the basolateral membrane, as visualised by fluorescence microscopy. Tubules were sensitive to the GC-A ligand ANP, and responded in a dose-dependent manner. Fluid secretion of tubules was elevated by ANP, and an EC_{50} value of 5×10^{-9} M was calculated from fluid secretion data. However, at high concentration of ANP (0.1 mM) fluid secretion was attenuated. This "inverted U" profile is similar to that observed when tubules are treated with exogenous cGMP (Davies *et al*, 1995). Other workers have shown that CAP_{2b} or cGMP are inhibitory in other species (Quinlan *et al*, 1997; Wiehart *et al*, 2002; Eigenheer *et al*, 2002), which suggests that cGMP acts as an anti-diuretic at high concentrations.

Tubules expressing GC-A under heat-shock control showed higher maximal rates of fluid secretion in response to ANP than tubules expressing GC-A in only one cell-type of the tubule main segment, implying that there was an additive effect of second messenger generation in both cell types. This data reveals a role for cGMP in stellate cells that is distinct from its function in principal cells. The levels of cGMP generated in response to ANP were directly proportional and a similar EC_{50} for ANP was calculated from cGMP data. Both fluid secretion and the subsequent generation of cGMP can be attenuated with the peptide inhibitor anantin, with an IC_{50} of 5×10^{-7} M.

Immunocytochemistry indicated that the cGMP generated by GC-A in response to ANP was localised to pools at the basolateral membrane, which suggests that there is a low diffusibilty of cGMP and that there is perhaps compartmentalisation of effectors. It may also suggest that localisation of cGMP at the basolateral membrane elicits a different response to the cGMP signal generated by a soluble guanylate cyclase. This could be achieved by tight

signalling complexes, which might include cGMP-dependent kinases or phosphodiesterases in order to achieve tight coordination of the signal.

Indeed, a major effector of cGMP, the cGMP-dependent kinase (cGK) was shown to be upregulated in response to ANP in GC-A transgenic tubules, expressing GC-A in either principal cells or stellate cells. Elevated kinase activity could potentially lead to phosphorylation of a huge range of substrates that would be able to contribute to stimulation of fluid secretion. It has previously been shown that tubules incubated with exogenous cGMP or with CAP_{2b} have increased V-ATPase activity (O'Donnell *et al*, 1996). This demonstrates that cGMP, or one of its effectors, acts to increase V-ATPase activity, suggesting this may be a target for phosphorylation by cGK.

Phosphodiesterase (PDE) activity in tubules expressing GC-A was examined, and it was found that tubules had elevated cGMP-dependent PDE activity in response to ANP. This indicates that there is a simple negative feedback loop which dampens the cGMP signal. In contrast, cAMP-hydrolysing phosphodiesterase activity was shown to decrease below basal activity. This suggests an element of cross-talk between cGMP and cAMP signal transduction pathways in tubules to downregulate cAMP hydrolysis. A plausible explanation is that a cAMP-hydrolysing PDE is inhibited by cGMP, as is the case with the mammalian PDE3 family. There was no significant elevation of cAMP levels in these tubules, however, which implies that the decrease in cAMP-dependent PDE activity would only be seen to affect tubules that had concurrent elevated adenylate cyclase activity, or that a cGMP-inhibited PDE has dual hydrolysing specificity for cGMP and cAMP. Tubules have been shown to express at least four distinct PDEs by RT-PCR, but none of these appear to be an orthologue of mammalian PDE3 by sequence similarity (Jonathan Day, personal communication). This does not rule out the possibilty that a cAMP-hydrolysing PDE is negatively regulated by a component of the

cGMP signalling cascade in a less direct manner. Indeed, tubules express a cAMP-hydrolysing PDE that is homologous to mammalian PDE8, which has unknown regulators.

The effect of cell-specific production of cGMP as a modulator of Ca^{2+} signalling was assayed. Tubules expressing GC-A in principal cells showed a biphasic elevation of $[Ca^{2+}]_i$, which is indicative of a calcium-induced calcium rise. Interestingly, ANP has been shown to stimulate L-type Ca^{2+} channel current in other systems (Barrett *et al*, 1991). These channels have also been shown to be important in regulation of fluid transport in Malpighian tubules (MacPherson *et al*, 2001). Furthermore, these channels have been localised to principal cells of tubules, and may explain why tubules expressing GC-A only in stellate cells do not respond to ANP with changes in $[Ca^{2+}]_i$. Therefore, it is likely that cGMP provides different mechanisms of action in different cell types of tubules, and opens the question of what its role may be in control of stellate cell function.

Cyclic nucleotide-gated ion channels have been shown to be expressed in Malpighian tubules (MacPherson *et al*, 2001), but are likely only to be present in principal cells. These ion channels are relatively non-selective, but have high conductance for Ca^{2+}, and may contribute to the rise in $[Ca^{2+}]_i$ that is seen in principal cells. Similarly, if these channels were present in stellate cells, an ANP-induced cGMP rise would likely cause an influx of Ca^{2+}, which has been shown not to occur. A possible role for cGMP in stellate cells could be regulation of Cl^- channels. This would, however be indirect, as the general classes of Cl^- channel are those activated by (i) intracellular cAMP, (ii) cell swelling, (iii) hyperpolarization and (iv) intracellular Ca^{2+}. Malpighian tubules have been shown to increase fluid secretion by elevated Cl^- conductance in response to stimulation of a G protein-coupled receptor that is coupled to PLC-activated release of Ca^{2+} through $InsP_3$ receptor channels in the ER. However, chloride channels may also be opened by cAMP, through phosphorylation of

the channel by cAK. Therefore, if cGMP or a cGMP-activated kinase could cross-activate cAK, chloride conductance may be increased. Both cAK assays and microelectrode measurements in tubules would need to be conducted in order to test this possibilty. It should be noted that it has previously been demonstrated that cyclic nucleotides elicit only a negligible effect on anion conductance (O'Donnell *et al*, 1996). This did not address stellate cell-specific increases in cyclic nucleotide levels, but only the effect that incubuation with exogenous cyclic nucleotides had on transepithelial potential, and therefore may not have unmasked this possible control of anion conductance. Cyclic nucleotides may only be transported into principal cells and, therefore, the ability to manipulate cyclic nucleotides in stellate cells is generating new results. This is accordingly the first demonstration that cGMP plays a role in stellate cells.

6.1.2 $5HT_{7Dro}$ and cAMP signalling.

Expression of $5HT_{7Dro}$ in tubules confers 5HT-stimulated elevation of fluid secretion, when expressed in either a principal cell- or stellate-cell specific manner. Production of cAMP is directly proportional to the concentration of 5HT used to stimulate tubules, with an EC_{50} of approximately 5×10^{-8} M. This can be attenuated by the $5HT_{7Dro}$ antagonist (+)-butaclamol, which has a calculated IC_{50} of approximately 2 μM.

This is the first demonstration that an extracellular ligand can stimulate a G protein-coupled receptor to signal *via* cAMP in *Drosophila* Malpighian tubules. As with cGMP, it has previously been demonstrated that exogenous cAMP does elevate fluid secretion through an increase in V-ATPase activity but has a negligible effect on anion conductance (O'Donnell *et al*, 1996). The same argument applies as it did for the role of cGMP in stellate cells: there are perhaps only cyclic nucleotide transporters on the basolateral membrane of

principal cells, and therefore the role of cyclic nucleotides in stellate cells remained unmasked. There are a number of important proteins that may be regulated by cAMP and cAMP-dependent kinase in tubules. Of course, the V-ATPase is already implicated as a major candidate for control by cAMP in *Drosphila* tubules (Coast *et al*, 2001). It has also been suggested that cAMP may stimulate a basolateral Na^+/K^+-ATPase directly, as addition of the Na^+/K^+-ATPase inhibitor ouabain to cAMP-stimulated tubules reduces fluid secretion rate slightly (Linton and O'Donnell, 1999). However, these are principal cell-specific modulations, and it is apparent from fluid secretion assays that cAMP produced specifically in stellate cells also acts to elevate fluid secretion rate.

The role of stellate cells is proposed to be mainly for the passage of water and Cl^-, as determined firstly by the simple architecture of these cells. More conclusively, however, is that both the receptor for Drosokinin and the subsequent elevated chloride conductance in response to activation of the receptor by Drosokinin are localised to the stellate cells (O'Donnell *et al*, 1998; Radford *et al*, 2002). This suggests chloride channels are good candidates for regulation by cAMP or cAK: tubule chloride channels have been shown to be activated by Ca^{2+} in response to Drosokinin (O'Donnell *et al*, 1998), but there are perhaps other chloride channels present, regulated in a different manner, which remain to be characterised. Additional routes for chloride flux have been proposed, which include K^+/Cl^- cotransport across the baslolateral membrane of principal cells (Linton and O'Donnell, 1999) and *via* a sodium-dependent Cl^-/HCO_3^- exchanger, also expressed on the basolateral membrane of tubules (Sciortino *et al*, 2001); regulation of these transporters may involve cAMP either directly or indirectly.

A second possible role for cAMP in stellate cells would be in the recruitment of water channels (aquaporins, AQP) to the apical and/or basolateral plasma membranes to increase tubule permeability. The elevation of cAMP by the

hormone vasopressin, with subsequent phosphorylation of mammalian AQP2, is necessary to recruit AQP2 from intracellular vesicles to apical membranes of renal collecting duct epithelial cells (Sabolic *et al*, 1992; Sabolic *et al*, 1995; Kuwahara *et al*, 1995; Nielsen *et al*, 1997). Interestingly, nitric oxide and ANP can both stimulate mammalian AQP2 insertion into membranes, which implies that this aquaporin can be regulated by cGMP as well as by cAMP (Bouley *et al*, 2000). It has also been shown that another aquaporin, AQP1, which was originally thought to be constitutively expressed on the apical and basolateral membranes of proximal tubules and thin descending limbs of Henle, is also regulated by arginine vasopressin (Yool *et al*, 1996; Patil *et al*, 1997; Han and Patil, 2000). Malpighian tubules have been shown to express six water transporters by RT-PCR (Lesley Cunliffe, personal communication), and are therefore possibly regulated by cyclic nucleotides or cyclic nucleotide dependent kinases. This would be an interesting idea to investigate in future, and using GC-A and $5HT_{7Dro}$ transgenic tubules would be ideal for doing so.

Phosphodiesterase activity is modulated in $5HT_{7Dro}$ tubules in response to 5HT, which highlights the importance of this enzyme in regulation of cyclic nucleotide signalling. The levels of intracellular cGMP are perhaps decreased very slightly in $5HT_{7Dro}$ tubules in response to 5HT, but not significantly. The increase in cAMP results in a significant elevation of cAMP-hydrolysing phosphodiesterase activity. Similarly, the slight decrease of cGMP below basal levels in response to 5HT corresponded to significantly elevated cGMP-hydrolysing phosphodiesterase activity. As discussed earlier, tubules express at least four different PDEs; two of which are likely to be cAMP-hydrolysing PDEs. These PDEs are *dunce* and a homologue to mammalian PDE8A, which specifically hydrolyses cAMP. However, there is also a PDE expressed in tubules homologous to PDE11A that is possibly a dual-hydrolysing PDE (Broderick *et al*, in preparation). Therefore, there is the possibility that the

elevation of cAMP- and cGMP-hydrolysing PDE activity is by this single PDE, which can be activated by elevation of $[cAMP]_i$. The regulators of these *Drosophila* PDEs are yet to be elucidated, and yet another possibility is that the cGMP-hydrolysing PDE found in tubules (homologous to mammalian PDE 6, which is regulated by light) is regulated by cAMP or cAK.

Kinase activity dependent on cAMP was not assayed due to time constraints, but is a major effector of cAMP in most cellular systems and is likely to be important in any signal transduction pathway that cAMP is involved in.

$[Ca^{2+}]_i$ levels were assayed in tubules expressing $5HT_{7Dro}$ cell-specifically. Stimulation of tubules expressing $5HT_{7Dro}$ in principal cells with 5HT resulted in a biphasic elevation of $[Ca^{2+}]_i$, whereas there was no change in tubules expressing $5HT_{7Dro}$ in stellate cells. This implies that the $5HT_{7Dro}$ receptor does not directly stimulate Ca^{2+} release into the cytosol *via* activation of PLC, as if this was the mechanism of elevation of $[Ca^{2+}]_i$, it would be seen in both cell types. The elevation observed in principal cells could either be due to downstream cross-talk, which does not take place in stellate cells, or that principal cells contain cyclic nucleotide gated channels that are not present in stellate cells.

RT-PCR analysis of G proteins was performed to determine which of the α, β and γ subunits were expressed in tubules. Unsurprisingly, fragments for all but one of the subunit genes were consistently amplified, which suggests there is great diversity in the signals received and transduced by tubules. This includes those that both stimulate and inhibit adenylate cyclase, and those which result in the mobilisation of Ca^{2+} from internal ER stores, as the relevant Gα subunits were detected. Of course, this experiment does not demonstrate that every cell in the tubule expresses every G protein, but is at least consistent with the results in this thesis.

6.1.3 $5HT_{1ADro}$ and Ca^{2+} signalling.

Flies expressing $5HT_{1ADro}$ in a cell-specific manner responded to 5HT in a dose-dependent manner in fluid secretion assays. The pharmacological profile of this receptor was similar when specifically expressed in either principal cells or stellate cells. The calcium response to 5HT stimulation was considerably larger when $5HT_{1ADro}$ was expressed ubiquitously than the response of principal- and stellate cell-specific calcium responses, indicating that these signals may have an additive response. However, it would be very interesting to determine the effects of modulating $[Ca^{2+}]_i$ levels in other regions of the tubule, for example initial or lower segments, using the appropriate GAL4 driver.

No variation in intracellular cGMP levels resulted from stimulation of $5HT_{1ADro}$ tubules with 5HT, suggesting that there is not cross-talk between these two signalling pathways, when $[Ca^{2+}]_i$ levels are elevated in tubules by this method. This highlights the need for very specific signals to trigger specific responses: the Ca^{2+} signal generated in principal cells did not eventually lead to elevated cGMP levels, as it does in CAP_{2b}-stimulated tubules, and implies that these Ca^{2+} signals are differentially recognised.

Interestingly, there was no reduction in basal levels of cAMP in tubules expressing $5HT_{1ADro}$ when stimulated with 5HT. However, this receptor has previously been demonstrated to inhibit adenylate cyclase activity. This leads to one of two possibilities: the $5HT_{1ADro}$ receptor couples with a G protein that leads to PLC activation, but not to adenylate cyclase inhibition in *Drosophila* epithelial cells; or more simply, under these conditions there is very little adenylate cyclase activity, and inhibition of this enzyme can only be unmasked when it is activated by forskolin.

6.2 Future work

At the end of chapters 3 and 4, cyclic nucleotide assays were conducted on brains from flies expressing GC-A and $5HT_{7Dro}$ respectively. These assays aimed to illustrate that the scope of this project is not restricted to Malpighian tubules, or indeed epithelia. In chapter 3, GC-A was expressed in brains by heat-shock, and this conferred sensitivity to ANP, with concurrent elevation of $[cGMP]_i$ in stimulated samples. Similarly, in chapter 4, the $5HT_{7Dro}$ receptor was expressed in brain, and this resulted in a slight elevation of 5HT above wild type control levels. This elevation could be potentiated with the addition of 5HT to samples. These transgenic lines are therefore suitable to study second messenger signalling in isolated tissues, and perhaps even to determine behavioural phenotypes in the living animal. There are, of course, limitations: ectopic expression of a receptor requires that the cells of interest do not normally respond to the cognate ligand. In the case of the $5HT_{7Dro}$ receptor, it would need to be established that the cell in which it is expressed does not normally respond to serotonin. This is not an issue for ectopic expression of GC-A, as *Drosophila* does not produce endogenous ANP, or any related peptide. However, this does present an additional limitation. The biochemistry and physiology of an isolated tissue expressing the transgenic receptor can be examined if the tissue is bathed in the appropriate ligand, but this is not useful if, for example, behavioural phenotypes were of interest. This could perhaps be overcome, either by injection of hormone/peptide into the haemolymph of live animals, or more ambitiously, by ectopic expression of the gene for the hormone/peptide in neurosecretory cells. Hormone delivery could then be under external control, allowing activation of the receptor transgene to be conditional.

6.3 Conclusions

Here, it has been demonstrated that transgenic receptors integrate effectively with endogenous machinery, and behave broadly as expected. This technique has also given some interesting insights to Malpighian tubule regulation. For example, intracellular modulation of cyclic nucleotides in stellate cells affects fluid secretion of tubules, presumably by controlling the permeability of these cells to water and/or chloride.

In conclusion, integrative physiology has combined genetics/transgenics with classical physiology, to create a powerful system for *in vivo* characterisation of receptors, and also for transgenic manipulation of cGMP, cAMP and Ca^{2+} in almost any cells of choice.

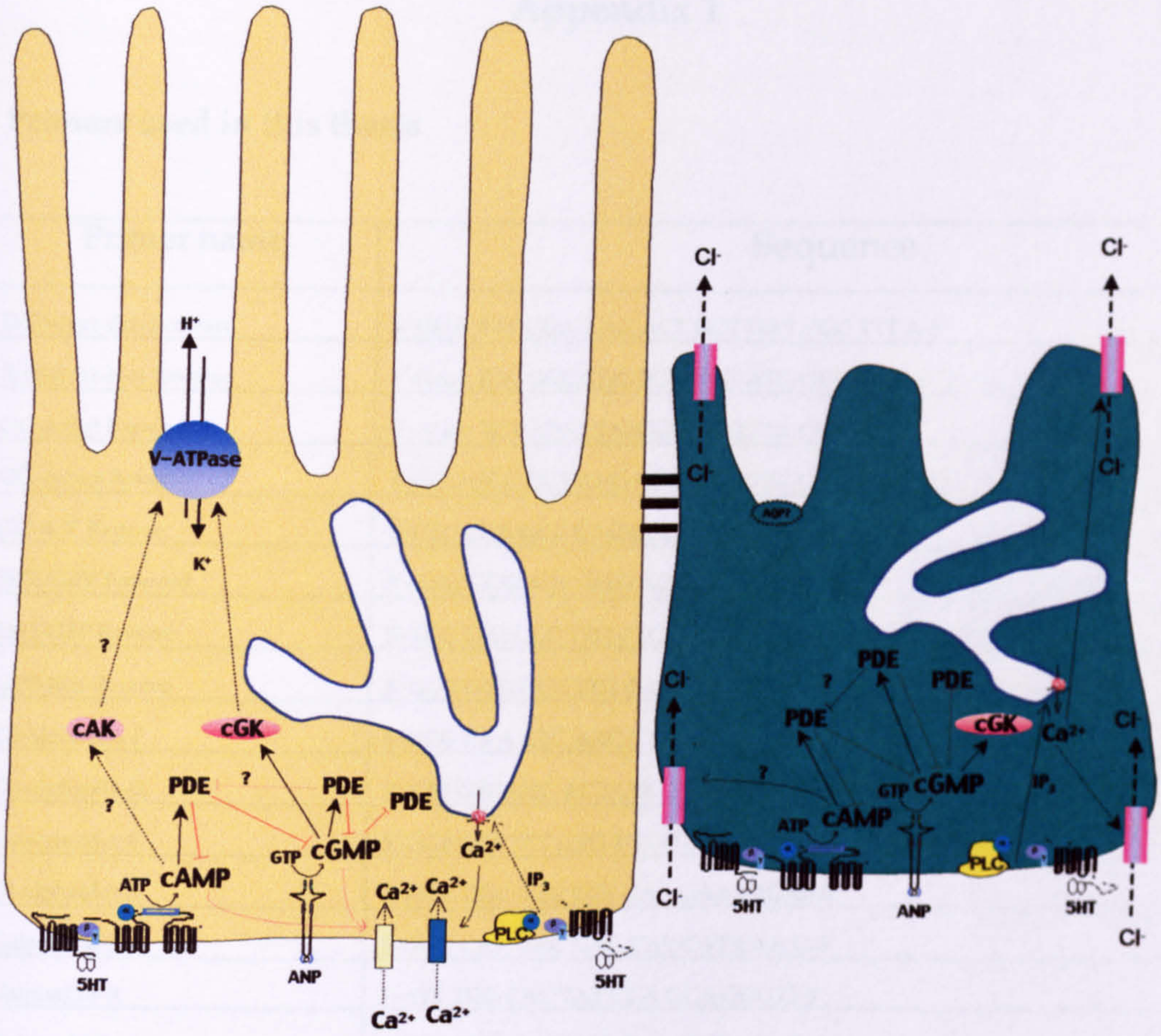

Figure 6.1: Summary of proposed intracellular signalling and crosstalk in Malpighian tubules

Elevation of intracellular cAMP ($[cAMP]_i$) in principal cells by activation of $5HT_{7Dro}$ with 5HT elevates fluid secretion, presumably by increasing V-ATPase activity (and possibly also through activation of basolateral ouabain-sensitive Na^+/K^+ -ATPase activity). A rise in intracellular Ca^{2+} is also seen, indicating that cyclic nucleotide-gated channels (CNG) are activated. Cyclic AMP- and cGMP-hydrolysing phosphodiesterase (PDE) activity is elevated in response to activation of $5HT_{7Dro}$, and completes a negative feedback loop.

Similarly, elevation of intracellular cGMP ($[cGMP]_i$) in principal cells by activation of GC-A with ANP elevates fluid secretion, increasing V-ATPase activity and inducing a rise in intracellular Ca^{2+}. There is also elevation of cGMP-dependent kinase (cGK) activity in response to ANP, implicating this enzyme in control of fluid secretion. ANP stimulation evokes modulations in PDE activity, increasing the rate of hydrolysis of cGMP in a negative feedback loop, but conversely decreases the rate of hydrolysis of cAMP.

Elevation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) in principal cells by activation of $5HT_{1ADro}$ with 5HT elevates fluid secretion *via* a calcium-induced calcium rise, which does not impinge on cyclic nucleotide signalling.

Elevation of $[cAMP]_i$ and $[cGMP]_i$ in stellate cells by activation of $5HT_{7Dro}$ and GC-A, respectively, elevate fluid secretion rate with no downstream activation of a Ca^{2+} signal, suggesting that CNGs are not found in this cell type. As there are no V-ATPases in stellate cells, this reveals a separate function for cyclic nucleotides in this cell type. This could be control of chloride channel activity, or an increase in permeability *via* aquaporin recruitment to the plasma membrane. PDE activity is elevated in response to $5HT_{7Dro}$ activation.

Appendix 1

Primers used in this thesis

Primer name	Sequence
5HT-dro1-Eco Forward	5′-GGA ATT CGA AAG ACT GCT GAT GGC TTT A-3′
5HT-dro1-Not Reverse	5′-GAA TGC GGC CGC TCG TGG ATG GCC TAG AGA AA-3′
GC-A-Bgl Forward	5′-AGA TCT ATG CCG GGC TCCCGA CGC GT-3′
GC-A-Not Reverse	5′-GCG GCC GCT CAG CCT CGA GTG CTA CAT C-3′
GC-A 5′ Reverse	5′-CTC CCA GCC CAG CCG TCG ATG CAG CG-3′
5HT/GFP Forward	5′-GGG GCG AGC GAG AGC TTT CTC ATG GTG AGC AAG GGC-3′
5HT-GFP Reverse	5′-TCC TCG CCC TTG CTC ACC ATG AGA AAG CTC TCC CTC-3′
GFP-Not-Reverse	5′-GCG GCC GCC TTG TAC AGC TCG TCC ATG-3′
Gs-alpha60A-F	5'-CGG CAA ATC AAC CAT AGT CAA GC-3'
Gs-alpha60A-R	5'-ATT GTG CTC ACT CGG TCC AGG AAG-3'
Gi-alpha65A-F	5'-CGT GTG TTT GTG TGT GTG TGC TTG-3'
Gi-alpha65A-R	5'-AGA GGC GTG TGT GAA GAA CTG G-3'
Galpha73B-F	5'-GGG CAA GAC GAC CAT CAT CAA G-3'
Galpha73B-R	5'-ATT TCG CAC TAT CCA GCA GGG G-3'
Galpha 2B-F	5′-GCA TCG GAA TCG GAA TCC ATA G-3′
Galpha 2B-R	5′-CAC GCA CTA AGC ACA AAA CGG-3′
cta-F	5′-CAA TGT CAT CAC GAA CGG CG-3′
cta-R	5′-TTT CCA GAT TCC CCA GCA CC-3′
Goalpha 47A-F	5′-CAA CCC ACC GCC TTT TTT TC-3′
Goalpha 47A-R	5′-TTA TGA CCG CCA CTG TCT GTG C-3′
CG17760 -Galpha-F	5'-GGC TTG TGA GGA AAG GCG TAT C-3'
CG17760 -Galpha-R	5'-CCA AAA ATC CCA GGA CCA ATA GC-3'
Galpha49B-F	5'-CCT CGG ATA GCG GAG TTA GTT TAG C-3'
Galpha49B-R	5'-AAC GAT GCC AGT GGT CTT GAC G-3'
Gbeta60B-F	5'-GAT GGC TTA GAA ACT GTG TGG TCG-3'
Gbeta60B-R	5'-TAC TGG CAG ATG CGG AAG GTC TTG-3'
Gbeta 8F-F	5′-CGA TGA GAT TCG TGG AGA CCT C-3′
Gbeta 8F-R	5′- GCA AAC AGG CGG CAT TTA CC-3′
Gbeta 76C-F	5′-ATG AAC CGC CCC AGA AGA ACA C-3′
Gbeta 76C-R	5′-TTA GAG CCA CAG ACG CAC TTG CTG-3′
Gbeta 5-F	5′-CAA GCG TCA CAT CAT CTC CTC G-3′
Gbeta 5-R	5′-AGG TGT TGC CCG TTT CGT TG-3′
Gbeta 13F-F	5′-GCA AAC ACA GGA CGA CGA CTA TG-3′
Gbeta 13F-R	5′-CGT TCT TTA GGG ACT CGG CTT C-3′
Ggamma1-F	5'-GTT TAG CAA GAT GGA TTA GGA CGC-3'

Ggamma1-R	5'-GAG TTT GTG TTT CTG GTT TCG GAC-3'
Ggamma30A-F	5'-AAA TGC CCT CAA CAT CAT CAG C-3'
Ggamma30A-R	5'-TTA GCC AAA GGA CGA ACA GGA G-3'
Sp 3	5′-GAG TAC GCA AAG CTT TAA CTA TGT-3′
Splac 2	5′-GAA TTC ACT GGC CGT CGT TTT ACA A-3′
Spep 1	5′-GAC ACT CAG AAT ACT ATT C-3′
P Lac 4	5′-ACT GTG CGT TAG GTC CTG TTC ATT GTT-3′
P Lac 1	5′-CAC CCA AGG CTC TGC TCC CAC AAT-3′
Plw 3-1	5′-TGT CGG CGT CAT CAA CTC C-3′
Pry 1	5′-CCT TAG CAT GTC CGT GGG GTT TGA AT-3′
Pry 2	5′-CCT GCC GAC GGG ACC ACC TTA TGT TAT T-3′
pUAST Forward	5′-GAA ATC TGC CAA GAA GT-3′
M13 (-20) Forward	5′-GTA AAA CGA CGG CCA G-3′
M13 Reverse	5′-CAG GAA ACA GCT ATG AC-3′
T7	5′-GTA ATA CGA CTC ACT ATA GGG C-3′
T3	5′-AAT TAACCC TCA CTA AAG GG-3′
PKC53e-F	5'-AGA CCA CAA AGA CTT TCT GCG G-3'
PKC53e-R	5'-GGC TTA AAT GGC GGT GCA C-3'
vha55-F	5′-AAT TCT CGA AAT TTG CAG TGC AC-3′
vha55-R	5′-GAA TTG GGG GTC CCT TGT CGA TG-3′

Appendix 2

Drosophila and *E. coli* media

Drosophila media

Standard growth medium (per litre of water)

10 g agar
15 g sucrose
30 g glucose
35 g dried yeast
15 g maize meal
10 g wheat germ
30 g treacle
10 g soya flour

Egg collection medium (per litre of water)

15 g Bacto-agar
26 g sucrose
52 g glucose
10 g dried yeast
100 ml grape juice

Escherichia coli growth media

L-broth (per litre of water)

10 g bacto-tryptone
5 g yeast extract
10 g NaCl

L-agar (per litre of water)

10 g bacto-tryptone
5 g yeast extract
10 g NaCl
15 g Bacto-agar

SOC broth

2% tryptone
0.5% yeast extract
10 mM NaCl
2.5 mM KCl
10 mM $MgCl_2$
10 mM $MgSO_4$
20 mM glucose

Appendix 3

Inverse PCR was performed with various transgenic fly lines. PCR products were sequenced and aligned against the *Drosophila* genome, to determine the chromosomal localisation of the P element insertions. A table summarising these locations, and the corresponding *Drosophila* BLAST searches are below.

Transgenic line	Chromosomal localisation
c42	100C-100D
hs::$5HT_{7Dro}$	2R 58E-59A
UAS::$5HT_{7Dro}$	3R 85B
hs::GC-A	?
UAS::GC-A	3L

P{GAL4}c42 chromosomal location

```
AC008310 : Drosophila melanogaster, chromosome 3R, region 100C-100D, BAC clone
           BACR16C04, complete sequence.  Last updated 22-MAR-2001
          Length = 159,528

 Minus Strand HSPs:

  Score = 3025 (459.9 bits), Expect = 5.9e-130, P = 5.9e-130
  Identities = 615/625 (98%), Positives = 615/625 (98%), Strand = Minus / Plus

Query:     710 TTCTTGCTCTCGCGCTGAGTTGTGAAAATTTCCNCAAGTTAAAATTAAATGAAAAAAGTG 651
               |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct: 145694 TTCTTGCTCTCGCGCTGAGTTGTGAAAATTTCCACAAGTTAAAATTAAATGAAAAAAGTG 145753

Query:     650 CAAAGGAAGCAGGCGAAATAGCGAATAGTAATTTCGGTTTAACAACTGCGGGCAGTGCAC 591
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 145754 CAAAGGAAGCAGGCGAAATAGCGAATAGTAATTTCGGTTTAACAACTGCGGGCAGTGCAC 145813

Query:     590 TAGTAACCCTTTAAATGTGATGAAAGAGTGGAGAAACTATTGGTGTTTCCAACGAATAAG 531
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 145814 TAGTAACCCTTTAAATGTGATGAAAGAGTGGAGAAACTATTGGTGTTTCCAACGAATAAG 145873

Query:     530 TTTAAACTTCTAATATAAACTGAATAAGGTGCTTATAATGTTTATTTAACATACATATAG 471
               |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct: 145874 TTTAAACTTCTAATACAAACTGAATAAGGTGCTTATAATGTTTATTTAACATACATATAG 145933

Query:     470 TGATAGTTTCAGAAGAACATAAATTCAAACTTAAAATACAATACGTACAAATATGTAATC 411
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 145934 TGATAGTTTCAGAAGAACATAAATTCAAACTTAAAATACAATACGTACAAATATGTAATC 145993

Query:     410 GTATACAAAACAGTGACGAGGAGCGAAAATGGAAGGCTGTATAGTAATTTAGGACCTTGT 351
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 145994 GTATACAAAACAGTGACGAGGAGCGAAAATGGAAGGCTGTATAGTAATTTAGGACCTTGT 146053

Query:     350 ATATGCGATATCTGCGATATAATCCCCCCAACCGAAGAATCCGCTCTGTCTCGCTCGCTC 291
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 146054 ATATGCGATATCTGCGATATAATCCCCCCAACCGAAGAATCCGCTCTGTCTCGCTCGCTC 146113

Query:     290 AGTATATCCGTCTCCTTTTAAAGGCGTACACGTACCCAAAGGGGGTGTAAAGCAGAGCGT 231
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 146114 AGTATATCCGTCTCCTTTTAAAGGCGTACACGTACCCAAAGGGGGTGTAAAGCAGAGCGT 146173

Query:     230 ATGTGTCCGTGTCCCCTGTAAAGTGATCTCTCCGCAGGCAACCGCCCACCCCTAAAACAT 171
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 146174 ATGTGTCCGTGTCCCCTGTAAAGTGATCTCTCCGCAGGCAACCGCCCACCCCTAAAACAT 146233

Query:     170 CCGCTCTTATCAGGCGCAGTGTGCGTGCGTGTATGTGTGCGCCTGGGGAGGGGGTGTGAG 111
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 146234 CCGCTCTTATCAGGCGCAGTGTGCGTGCGTGTATGTGTGCGCCTGGGGAGGGGGTGTGAG 146293

Query:     110 TGAGACAGCGATATGATTGAAGGGC 86
               |||| ||||| ||| | || ||
Sbjct: 146294 TGAG-CAGCGG-ATGTCGGCAGAGC 146316
```

P{hs::5HT$_{7Dro}$} chromosomal location

AC008350 : Drosophila melanogaster, chromosome 2R, region 58E-59A, BAC clone BACR11M08, complete sequence. Last updated 10-MAR-2001
Length = 169,534

Minus Strand HSPs:

Score = 2885 (438.9 bits), Expect = 1.3e-123, P = 1.3e-123
Identities = 599/617 (97%), Positives = 599/617 (97%), Strand = Minus / Plus

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Query:   865 TGAGAAGNT-AGAAATAAGG-GCAGAATATCCGCAAAGCTCCTAATTTTATGACATAACT 808
             ||||| | | ||| |||||| ||||||||||||||||||||||||||||||||||||||
Sbjct: 17704 TGAGA-GCTGAGAGATAAGGTGCAGAATATCCGCAAAGCTCCTAATTTTATGACATAACT 17762

Query:   807 TAAGGGCTGACGTCTTTACGAG-CGGCTCAAACCAATTCCAATAAATCCCCCAGACGCAT 749
             |||||||||||||||||||||| |||||||| |||| |||||||||||||| ||||||||
Sbjct: 17763 TAAGGGCTGACGTCTTTACGAGACGGCTCAAGCCAAATCCAATAAATCCCC-AGACGCAT 17821

Query:   748 AAACG-GGCTAGTGC-TGCGAAGCACTATGCATATGCGTAGCACGGCCATTCGCAGCACT 691
             ||||| ||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 17822 AAACGTGGCTAGTGCCTGCGAAGCACTATGCATATGCGTAGCACGGCCATTCGCAGCACA 17881

Query:   690 GCACAATAATGGCATAATAACAAGCAATTATCGTTATAATAGGCACCATTCCGGCCACGC 631
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
Sbjct: 17882 GCACAATAATGGCATAATAACAAGCAATTATCGTTATAATAGGCACCATTCCGGCTACGC 17941

Query:   630 CGGAGAACATAACCGTTACGTTCCTCACGCCCACGGCGGTCCGGGTGTCGTGGCAGACGC 571
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 17942 CGGAGAACATAACCGTTACGTTCCTCACGCCCACGGCGGTCCGGGTGTCGTGGCAGACGC 18001

Query:   570 AAATCGATCTGAAGGCACATCCCATCGAGAAGTACATCGTGACGTACAAGCCAACGGACG 511
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 18002 AAATCGATCTGAAGGCACATCCCATCGAGAAGTACATCGTGACGTACAAGCCAACGGACG 18061

Query:   510 ACAGGTAGGTACCACTAACAAAGCTAGAACTCAATCCGCCGGGCCACTTGACTGTCCAAA 451
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 18062 ACAGGTAGGTACCACTAACAAAGCTAGAACTCAATCCGCCGGGCCACTTGACTGTCCAAA 18121

Query:   450 TGACGGGCAAAGCACACTCACTTACCATTCAGTTGACGGGCTAAAAGGGACTTTGAGGTT 391
             |||||||||||||||||||||||||||||||| |||||||||||||||||||| |||
Sbjct: 18122 TGACGGGCAAAGCACACTCACTTACCATTCAGTTGGCGGGCTAAAAGGGACTTTGACGTT 18181

Query:   390 CCGACGGGTTCCGACTGCACGGCCAAAAGCATTGGCCACATTTTGTTAACGGGAAGGCTT 331
             |||||||||||||||||||||||||| |||||||||||||||||||||||||||||||||
Sbjct: 18182 CCGACGGGTTCCGACTGCACGGCCAAAACCATTGGCCACATTTTGTTAACGGGAAGGCTT 18241

Query:   330 CATTAGATTCATTAGATTCATTAATAATGTATATAATTACATGCTGATTAGTTGGCTACT 271
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 18242 CATTAGATTCATTAGATTCATTAATAATGTATATAATTACATGCTGATTAGTTGGCTACT 18301

Query:   270 TAACTGTTTNTAAATTA 254
             ||| ||||| |||||||
Sbjct: 18302 TAAATGTTTCTAAATTA 18318
```

P{UAS::5HT$_{7Dro}$} chromosomal location

AC009512 : Drosophila melanogaster, chromosome 3R, region 85B-85B, BAC clone BACR01F13, complete sequence. Last updated 06-SEP-2001
Length = 167,467

Plus Strand HSPs:

Score = 2808 (427.4 bits), Expect = 3.8e-120, P = 3.8e-120
Identities = 570/579 (98%), Positives = 570/579 (98%), Strand = Plus / Plus

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Query:     1 CGATTGAATATTGAACCCCGCGGTGCGTTTCTCCTCTCGCAGCCAATCATCGTTTCGGC 60
             |||||||||||||||||||||||||||||||||||||||||||||| ||||||| ||||||||
Sbjct: 35828 CGATTGAATATTGAACCCCGCGGTGCGTTTCTCCTCTCGCAGCCGATCATCGCTTTCGGC 35887

Query:    61 GCGACTCTTTTTTCCTAGTGCCCATGCACGGCGGCATCTGGACACTGTGCATTGATCTGC 120
             |||||||| ||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 35888 GCGACTCTGTTTTCCTGGTGCCCATGCACGGCGGCATCTGGACACTGTGCATTGATCTGC 35947

Query:   121 CCATTCAGCAGCTGCAGGAACTGCGGCGCAACCCCAAGTTCCCACGCGGTGCTCCTCCGT 180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 35948 CCATTCAGCAGCTGCAGGAACTGCGGCGCAACCCCAAGTTCCCACGCGGTGCTCCTCCGT 36007

Query:   181 GTGTAAACTACCTGGCCGGCTCAATGGAAAACGCACGAGGCGAGGAGCAGCGCAACGACN 240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 36008 GTGTAAACTACCTGGCCGGCTCAATGGAAAACGCACGAGGCGAGGAGCAGCGCAACGACT 36067

Query:   241 GGCAGCACAGTGAGTCACAGGTCACTCTGCAGCCGCATCTGAGTACGTATTCAGGGGCAG 300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 36068 GGCAGCACAGTGAGTCACAGGTCACTCTGCAGCCGCATCTGAGTACGTATTCAGGGGCAG 36127

Query:   301 CCGGTTTGTTAATCCCAGTCCCAGATCCACATCCGCTCTACTAACTTTCTGGGGGAAATC 360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 36128 CCGGTTTGTTAATCCCAGTCCCAGATCCACATCCGCTCTACTAACTTTCTGGGGGAAATC 36187

Query:   361 TCCCGTATTTATTGTGTACACTTTTTGCCTTCCTACTTAGTTTTTCTTTTAATACAAAAC 420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 36188 TCCCGTATTTATTGTGTACACTTTTTGCCTTCCTACTTAGTTTTTCTTTTAATACAAAAC 36247

Query:   421 ATTATTTTATGTACGCACTTAGCACTAATTGTGATTGTAAACTTACAGACTTACAGAGAA 480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 36248 ATTATTTTATGTACGCACTTAGCACTAATTGTGATTGTAAACTTACAGACTTACAGAGAA 36307

Query:   481 TTTTGGTAGAAAACAGCAAACAAGTAGCATAAATATGTAGGTTCGAGGTATAAGACAGTT 540
             |||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct: 36308 TTTTGGTAGAAAACAGCAAACAAGTAGCATAAATATGTAGGTTCGAGGTATAGGACAGTT 36367

Query:   541 GAATGGTGTTTAATGCGTGNGGGGTTGGTT-AAGTAGCC 578
             |||||||||||||||||| ||| ||||||| ||||||||
Sbjct: 36368 GAATGGTGTTTAATGCGTGTGGG-TTGGTTTAAGTAGCC 36405
```

P{hs::GC-A} chromosomal location

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>gb|M59315|DRORUDPEB Drosophila melanogaster rudimentary gene with 3' P element
          insertion site.
        Length = 964

  Minus Strand HSPs:

 Score = 650 (103.6 bits), Expect = 7.9e-24, P = 7.9e-24
 Identities = 134/138 (97%), Positives = 134/138 (97%), Strand = Minus / Plus

Query:   183 CTTAGACCATGATGAAATAACATAAGGTGGTCCCGTCGGCAAGAGACATCCACTTAACGT 124
             ||| | ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   224 CTTCG-CCATGATGAAATAACATAAGGTGGTCCCGTCGGCAAGAGACATCCACTTAACGT 282

Query:   123 ATGCTTGCAATAAGTGCGAGTGAAAGGAATAGTATTNTGAGTGTCGTATTGAGTCTGGGT 64
             ||||||||||||||||||||||||||||||||||| |||||||||||||||||||| ||
Sbjct:   283 ATGCTTGCAATAAGTGCGAGTGAAAGGAATAGTATTCTGAGTGTCGTATTGAGTCTGAGT 342

Query:    63 GAGACAGCGATATGATTG 46
             ||||||||||||||||||
Sbjct:   343 GAGACAGCGATATGATTG 360
```

P{UAS::GC-A} chromosomal location

```
AC010036 : Drosophila melanogaster 3L BAC RP98-7K20 (Roswell Park Cancer
           Institute Drosophila BAC Library) complete sequence.  Last updated
           27-DEC-2001
         Length = 190,365

  Minus Strand HSPs:

 Score = 2041 (312.3 bits), Expect = 1.7e-85, P = 1.7e-85
 Identities = 427/449 (95%), Positives = 427/449 (95%), Strand = Minus / Plus

Query:   446 CACCCAAGGCTCTGCTC-CCA-CAATTATAAATACCACCAGAAATTATAATGCACAGAGT 389
             | | ||| |||| | |  ||| ||||||||||||| || ||||||||||||||||||||||
Sbjct:  5824 CCCACAACGCTCAGATGTCCAACAATTATAAATAACAACAGAAATTATAATGCACAGAGT 5883

Query:   388 CTTCAAATCGAATCGAAATGAAAACGCAA-CGAAGGAAATGCAACAATTGCAGGGGCCTC 330
             |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct:  5884 CTTCAAATCGAATCGAAATGAAAACGCAAACGAAGGAAATGCAACAATTGCAGGGGCCTC 5943

Query:   329 CGATTTCGACTTCAACTTTGAATTTGGACTCGGATTTGAATCGCGGCTTCGAAGAGCAAA 270
             ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  5944 CGATTTCGAATTCAACTTTGAATTTGGACTCGGATTTGAATCGCGGCTTCGAAGAGCAAA 6003

Query:   269 TTGCATAATTATTGCTACAAATGAAGCTGAGAAAGTGGTACCGTTTGAATCGTGATAAAG 210
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  6004 TTGCATAATTATTGCTACAAATGAAGCTGAGAAAGTGGTACCGTTTGAATCGTGATAAAG 6063

Query:   209 CGATAATTTTCCCCCTCACCGCCCAACGCAAAGAGCCAATGAAAATGCATTTCACTTTTC 150
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  6064 CGATAATTTTCCCCCTCACCGCCCAACGCAAAGAGCCAATGAAAATGCATTTCACTTTTC 6123

Query:   149 AGGTTAACAAACAAAACAATGCCGACTGTGTTATTATGAAATCTCGACAGTCACAATNNN 90
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  6124 AGGTTAACAAACAAAACAATGCCGACTGTGTTATTATGAAATCTCGACAGTCACAATAAA 6183

Query:    89 NNNNNNNGGGAAAACAACCGAAATCGCCCACGCGAAAATAGCTATAAATTCAAAGATGCA 30
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  6184 AAAAAA-GGGAAAACAACCGAAATCGCCCACGCGAAAATAGCTATAAATTCAAAGATGCA 6242

Query:    29 AAAAGATGCAATTTTTTGTTGCCAATATT 1
             ||||||||||||||||||||||||||||||
Sbjct:  6243 AAAAGATGCAATTTTTTGTTGCCAATATT 6271
```

Appendix 4

Solutions for use in Western blotting

Running buffer

1 M Glycine
1 M Tris base
10 % SDS

Transfer buffer

1 M Glycine
1 M Tris base
20 % methanol

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