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Agonist-specific coupling of a cloned *Drosophila* octopamine/tyramine receptor to multiple second messenger systems

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A cloned seven transmembrane-spanning *Drosophila* octopamine/tyramine receptor, permanently expressed in a Chinese hamster ovary cell line, both inhibits adenylate cyclase activity and leads to the elevation of intracellular Ca^{2+} levels by separate G-protein-coupled pathways. Agonists of this receptor (octopamine and tyramine), differing by only a single hydroxyl group in their side chain, may be capable of differentially coupling it to different second messenger systems. Thus, a single receptor may have a different pharmacological profile depending on which second messenger system is used to assay its efficacy.

Key words: *Drosophila*/G-protein-coupled receptors/octopamine/second messengers/tyramine

Introduction

A single agonist can modulate a range of second messenger systems by activating different G-protein-coupled receptor subtypes (Bylund, 1992; Hosey, 1992), but recently the use of cloned receptors has shown that single receptor subtypes can be directly linked to multiple second messenger pathways (Thompson, 1992). Thus, the cloned muscarinic-M2 (Lai *et al.*, 1991) and α_2-adrenergic (Cotecchia *et al.*, 1990) receptors both stimulate phosphoinositide hydrolysis and inhibit adenylate cyclase activity, whilst the cloned receptors for thyrotropin (Van Sande *et al.*, 1990), calcitonin (Chabre *et al.*, 1992), parathyroid hormone (Abou-Samra *et al.*, 1992) and the three classes of tachykinin receptor (Mitsuhashi *et al.*, 1992; Nakajima *et al.*, 1992) all stimulate both pathways. Here we report that a cloned seven transmembrane-spanning *Drosophila* octopamine/tyramine receptor (Arakawa *et al.*, 1990; Saudou *et al.*, 1990; Robb *et al.*, 1991), permanently expressed in a Chinese hamster ovary (CHO) K1 cell line, both inhibits adenylate cyclase activity and leads to the elevation of intracellular Ca^{2+} levels by separate G-protein-coupled pathways. Thus, this receptor is like the $OCTOPAMINE_1$ subclass of receptor that induces an elevation in intracellular Ca^{2+} levels, rather than the $OCTOPAMINE_2$ subclass that activates adenylate cyclase activity (Evans, 1981, 1984a,b). However, tyramine is about two orders of magnitude more potent than octopamine when assayed by direct binding or by depression of cyclic AMP levels, whereas octopamine is slightly more potent or faster in causing a transient elevation of cytosolic Ca^{2+}. Thus, agonists of this cloned receptor differing by only a single hydroxyl group in their side chain may be capable of differentially coupling it to different second messenger systems, a finding that could alter our current concepts of receptor pharmacology. A preliminary report of some of the information in this paper has been published previously (Evans and Robb, 1993; Robb *et al.*, 1994).

Results

Radioligand binding studies

Radioligand binding studies using [^{3}H]yohimbine, the α_2-adrenergic antagonist, were performed on membranes prepared from CHO-K1 cells transfected with a putative octopamine receptor cloned from *Drosophila* (Arakawa *et al.*, 1990). Table IA indicates that tyramine, the non-β-hydroxylated (i.e. side chain) precursor of octopamine, showed the highest agonist affinity for the receptor in these membranes. The phenylethylamines, β-phenethylamine and DL-β-hydroxyphenylethylamine (also known as RS-phenylethanolamine), which are biogenic amines lacking any ring hydroxyl groups, were more potent agonists than the enantiomers of the positional isomers of synephrine. These, in turn, were more potent than DL-*p*-octopamine (also known as *RS*-octopamine) or any of the enantiomers of the positional isomers of octopamine. An important novel finding of the present study was that the receptor binding showed little or no stereoselectivity between the (+)- and (−)-enantiomers of the most potent structural isomers of octopamine and synephrine. Nevertheless, the formamidine pesticides demethylchlordimeform and amitraz were also potent agonists, as they were at insect octopamine receptors (Evans and Gee, 1980).

To determine if these novel receptor properties were due to the expression of the *Drosophila* receptor in a vertebrate cell line, we also examined the properties of [^{3}H]yohimbine binding to membranes prepared from *Drosophila* heads (Jackson *et al.*, 1984). This binding exhibited similar properties to that of the cloned receptor expressed in CHO cells (Table IB) with tyramine again being approximately two orders of magnitude more potent as an agonist than DL-*p*-octopamine and the binding also lacking any stereoselectivity.

Attenuation of cyclic AMP levels

A similar pharmacological profile for the cloned *Drosophila* receptor to that described above in the radioligand binding studies was also obtained in studies of its ability to attenuate forskolin-stimulated cyclic AMP levels in the transfected CHO cells (Arakawa *et al.*, 1990), with a rank order of potency for the biogenic amines of tyramine > DL-*p*-synephrine > DL-*p*-octopamine (Figure 1A). In contrast to

Table I. Inhibition of [^{3}H]yohimbine binding

	K_i (μM)	*n*
A. Cloned Drosophila aminergic receptor in CHO membranes		
Yohimbine	0.012 ± 0.002	9
Tyramine	1.4 ± 0.4	3
β-Phenethylamine	10.2 ± 7.8	3
DL-β-Hydroxyphenylethylamine	18.0 ± 1.9	3
(+)-*p*-Synephrine	45.3 ± 8.1	3
(−)-*p*-Synephrine	50.6 ± 17.3	3
(−)-*o*-Octopamine	124.1 ± 11.1	4
DL-*p*-Octopamine	129.2 ± 23.4	4
Dopamine	137.9 ± 28.2	4
(−)-*p*-Octopamine	152.2 ± 43.0	4
(+)-*o*-Octopamine	186.8 ± 7.1	4
(+)-*p*-Octopamine	190.8 ± 45.9	4
(−)-*m*-Octopamine	334.4 ± 74.1	4
(+)-*m*-Octopamine	>1000	4
Demethylchlordimeform	1.17	2−6
Amitraz	2.53	1−3
B. Drosophila head membranes		
Yohimbine	0.0046 ± 0.0012	6
Phentolamine	0.35 ± 0.07	5
Tyramine	0.38 ± 0.13	3
Prazosin	3.5 ± 0.7	3
DL-*p*-Octopamine	62.1 ± 9.5	16
(−)-*p*-Octopamine	89.1 ± 14.8	6
(+)-*p*-Octopamine	57.4 ± 13.2	6

The inhibition of [^{3}H]yohimbine binding by various ligands was measured in membranes prepared from CHO cells transfected with a putative cloned *Drosophila* octopamine receptor (A) and from a *Drosophila* head preparation (B).

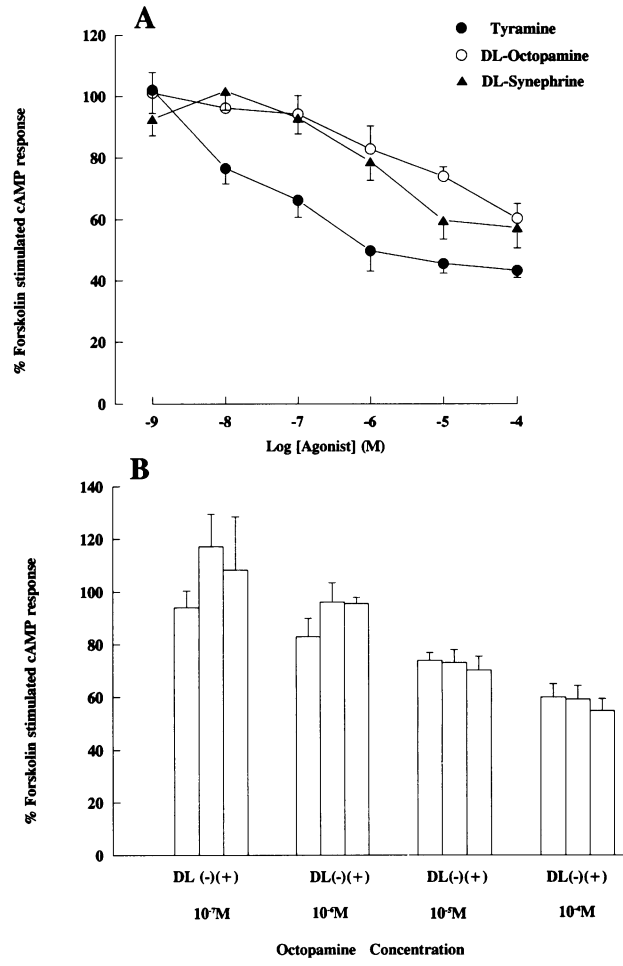

Fig. 1. The effect of biogenic amines on cyclic AMP levels in CHO-K1 cells expressing the *Drosophila* aminergic receptor. (**A**) Dose−response curves for the effects of tyramine, DL-synephrine and DL-*p*-octopamine. (**B**) Comparison of the dose responsiveness of the effects of (−)- and (+)-*p*-octopamine with that of DL-(+/−)*p*-octopamine.

octopamine receptors, the cloned receptor again lacked any stereoselective responses to the (−)- or (+)-enantiomers of *p*-octopamine (Figure 1B).

Initiation of transient intracellular Ca^{2+} signal

Since OCTOPAMINE$_1$ receptors mediate their effects via an elevation of intracellular Ca^{2+} levels (Evans, 1984b), we tested the ability of the cloned *Drosophila* receptor to raise intracellular Ca^{2+} levels in single transfected CHO cells (Figure 2). A Ca^{2+} transient, whose shape was dose dependent, was produced in response to the application of either octopamine or tyramine. Application of either 100 μM DL-*p*-octopamine or tyramine produced a transient (~30 s) elevation in $[Ca^{2+}]_i$ despite the continued presence of the agonist, a response seen in 95% of all cells challenged (Figure 2A and B). Equally, a sub-maximal (1 μM) dose of either DL-*p*-octopamine or tyramine also produced a transient rise in $[Ca^{2+}]_i$ that was slower in onset and had a longer recovery time (Figure 2A and B). The lag time before the onset of the Ca^{2+} response (Figure 2D) and the time to peak $[Ca^{2+}]_i$ (Figure 2E) decreased over the range 0.1−100 μM for both DL-*p*-octopamine and tyramine. Previous single-cell studies using different G-protein-coupled receptors on other cell types have shown a similar relationship between ligand concentration and the latency of the response (Millard *et al.*, 1988; Monck *et al.*, 1988; Corps *et al.*, 1989). Interestingly, peak $[Ca^{2+}]_i$ achieved in some cells could be similar in response to either 0.1 or 100 μM DL-*p*-octopamine (Figure 2F), and the number of cells responsive to DL-*p*-octopamine and tyramine increased with increasing doses of the agonist (Robb *et al.*, 1991). A similar intercellular heterogeneity of $[Ca^{2+}]_i$ signals has been reported in HeLa cells, where Ca^{2+} release was found to occur in a 'quantal' manner (Bootman *et al.*, 1992). The effects of both 100 μM DL-*p*-octopamine and tyramine were blocked in the presence of yohimbine at concentrations as low as 5 μM (n = 37). Surprisingly, the dose−response curves for the peak $[Ca^{2+}]_i$ responses achieved by tyramine and DL-*p*-octopamine were almost superimposable (Figure 2F). In contrast to the binding studies and adenylate cyclase inhibition studies, tyramine was not two orders of magnitude more potent than DL-*p*-octopamine. However, since the peak $[Ca^{2+}]_i$ response can vary substantially between individual cells, it may not reflect the efficacy of the interaction of the receptor with the second messenger system. The kinetics of the Ca^{2+} response (Figure 2D and E) may be a better reflection of this interaction and suggest that DL-*p*-octopamine may be a more effective agonist than tyramine for this response, since it consistently produces effects with a shorter lag time and a faster time to peak than tyramine. Thus, the efficacy of coupling of this cloned *Drosophila* aminergic receptor to different second messenger systems varies with the agonist used.

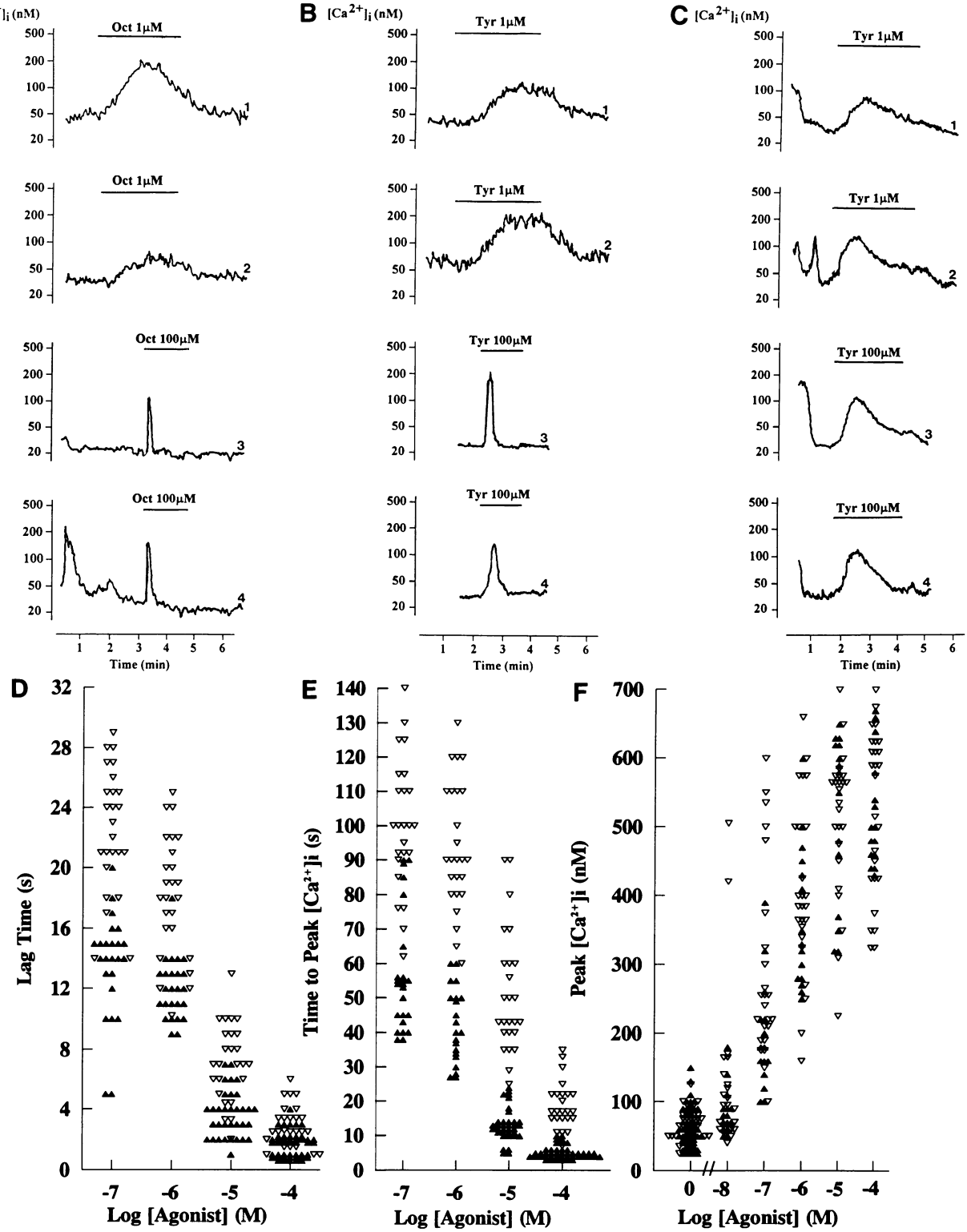

Fig. 2. The effects of DL-*p*-octopamine (Oct) and tyramine (Tyr) on intracellular Ca^{2+} levels in individual CHO-K1 cells expressing the *Drosophila* aminergic receptor. (**A**) Examples of the transient elevations in $[Ca^{2+}]_i$ induced by pulses of 1 or 100 μM DL-*p*-octopamine (horizontal line) in four different cells (1–4). In these cells, the pre-stimulus levels of $[Ca^{2+}]_i$ were variable. In ~40% (22/57) of the cells, $[Ca^{2+}]_i$ was in the range 30–100 nM and remained relatively constant (e.g. cell 3). In the remaining cells, asynchronous Ca^{2+} transients were observed in which $[Ca^{2+}]_i$ fluctuated from 50 to 500 nM (e.g. cell 4). (**B**) Examples of the transient elevations in $[Ca^{2+}]_i$ induced by 1 or 100 μM pulses of tyramine (horizontal line) in four different cells (1–4) from the same passage as the cells in (A). (**C**) Examples of the effects of the transient elevations in $[Ca^{2+}]_i$ induced by 1 or 100 μM pulses of tyramine (horizontal line) in four different transfected CHO cells (1–4) pretreated with 200 mg/ml of pertussis toxin (Sigma) at 37°C for 18 h. These cells are from the same batch as those shown in Figure 3D. (**D**) Dose–response plots for the effect of DL-*p*-octopamine (▲) and tyramine (▽) on lag periods before agonist-induced changes in $[Ca^{2+}]_i$ levels. (**E**) Dose–response plots for the effect of DL-*p*-octopamine (▲) and tyramine (▽) on time to peak changes in $[Ca^{2+}]_i$. (**F**) Dose–response plots for the effect of DL-*p*-octopamine (▲) and tyramine (▽) on peak $[Ca^{2+}]_i$ levels. The number of cells responsive to octopamine and tyramine increased with increasing doses of the agonist. Thus, at 0.1 μM DL-*p*-octopamine, 48% of the cells were responsive (n = 22), at 1 μM 62% responded (n = 25), at 10 μM 75% responded (n = 33), whilst at 100 μM 95% responded (n = 25). In (D), (E) and (F), each point represents the value obtained in a single cell.

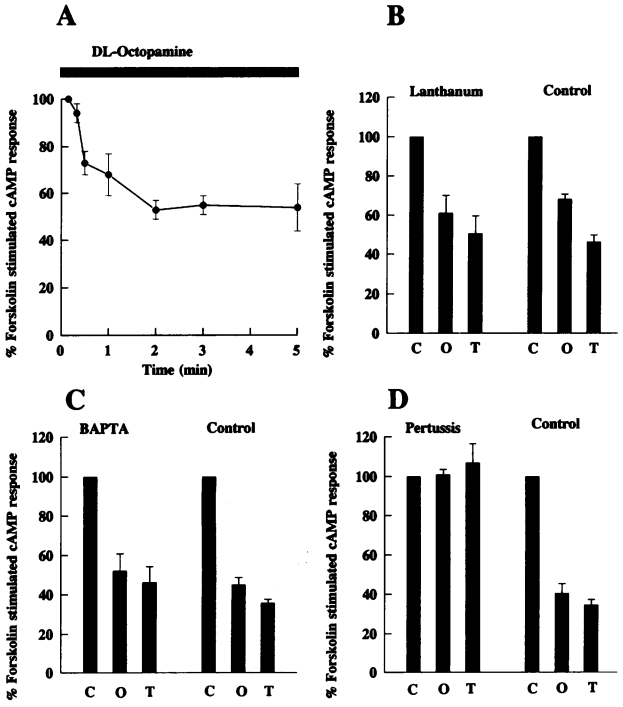

Fig. 3. The effects of biogenic amines on cyclic AMP levels in CHO-K1 cells expressing the *Drosophila* aminergic receptor. The experiments were performed as described in the legend to Figure 1. (**A**) shows the time course of the response to a prolonged pulse of 100 μM DL-*p*-octopamine. (**B**) shows the effects of 20 min incubations in the presence of 10 μM DL-*p*-octopamine (O) or tyramine (T) in the presence and absence of 75 μM lanthanum. Our results (not shown) indicate that lanthanum concentrations of ≥75 μM almost completely block the Ca^{2+} transients induced in the transfected CHO cells by both 100 μM DL-*p*-octopamine and tyramine. For example, 75 μM lanthanum reduces the peak rise in $[Ca^{2+}]_i$ from 525 ± 24 mM (n = 25) to 118 ± 9 mM (n = 25) at 100 μM DL-*p*-octopamine. (**C**) shows the effects of 20 min incubations in the presence of 100 μM DL-*p*-octopamine (O) or tyramine (T) in the presence and absence of 35 μM BAPTA. Initial experiments using BAPTA (not shown) indicate that concentrations of ≥35 μM block the Ca^{2+} transients induced in the transfected CHO cells by both 100 μM DL-*p*-octopamine and tyramine. (**D**) shows the effect of 100 μM DL-*p*-octopamine (O) and tyramine (T) after pretreatment of the transfected CHO cells with 200 ng/ml of pertussis toxin (Sigma) at 37°C for 18 h and under control conditions. The results were obtained from the same batch of cells used in Figure 2C. Data represent the average ± SEM of at least three separate experiments performed in duplicate and C represents the non-drug-treated control in all experiments.

Receptor coupling to second messenger systems

We have also examined whether the coupling of this cloned *Drosophila* aminergic receptor to the multiple effector systems is direct or whether the changes in cyclic AMP levels could be secondary to changes in intracellular Ca^{2+} levels, as has been shown to be the case for the activation of bradykinin receptors in NCB-20 cells (Boyajian *et al.*, 1991). In contrast to the transient nature of the receptor-induced changes in Ca^{2+} levels in the transfected cells when exposed to either tyramine or octopamine (Figure 2A and B), the inhibition of forskolin-stimulated cyclic AMP levels by 100 μM DL-octopamine (Figure 3A) or tyramine (not shown) was of a much slower time course, peaking after 2 min of exposure to the agonist. The response showed no desensitization and continued for as long as the cells were exposed to the agonist. Thus, the lag time for an observed change in cyclic AMP levels lies between 10 and 20 s after the start of the exposure of the cells to octopamine, a time corresponding to the peak of the Ca^{2+} response, suggesting that a maintained Ca^{2+} signal is not required to maintain the attenuation of the forskolin-induced cyclic AMP levels.

To rule out the possibility that receptor activation triggers either an influx of Ca^{2+} into the CHO cells, or a release of Ca^{2+} from intracellular stores, which then initiates a secondary change in cyclic AMP levels, experiments were performed in the presence of lanthanum (75 μM) to block Ca^{2+} entry and in the presence of the cell permeant Ca^{2+} chelator 1,2-bis-(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) (Lew *et al.*, 1982) (35 μM) to buffer intracellular Ca^{2+} levels (Figure 3B and C). In both cases, the effects of tyramine and octopamine on forskolin-stimulated levels of cyclic AMP were unaffected, although the Ca^{2+} transients were blocked, suggesting that this effect is not dependent upon Ca^{2+} influx or Ca^{2+} release from internal stores.

The octopamine- (Arakawa *et al.*, 1990) and tyramine-mediated attenuation of forskolin-induced increases in cyclic AMP levels in the transfected CHO cell line were blocked by pretreatment of the cells with pertussis toxin (Figure 3D). In contrast, in batches of cells taken from the same pertussis toxin-pretreated population the effects of 1 and 100 μM tyramine (Figure 2C) and octopamine (not shown) on Ca^{2+} levels were not blocked. Figure 2C shows that the responses to 1 μM tyramine were identical in pertussis toxin-treated cells and controls (Figure 2B), whilst the Ca^{2+} transients produced by 100 μM tyramine in the pertussis toxin-pretreated cells were also identical to those in controls (Figure 2B), except for a prolongation of the recovery phase.

Discussion

The cloned *Drosophila* aminergic receptor, expressed in the CHO-K1 cell line (Arakawa *et al.*, 1990) and used in the present study, represents a novel class of receptor despite sharing some pharmacological similarities with the $OCTOPAMINE_1$ subtype of receptor from locust skeletal muscle (Evans, 1981; Arakawa *et al.*, 1990). This finding is in agreement with those from an independent isolation of the same gene expressed in a different vertebrate cell line (Saudou *et al.*, 1990). An important novel finding of the present study was that the receptor expressed in the CHO cell line showed little or no stereoselectivity between the (+)- and (−)-enantiomers of the most potent structural isomers of octopamine and synephrine. This contrasts with previous studies on the pharmacological characterization of other insect octopamine receptors using physiological and biochemical studies, where the (−)-isomers were much more potent than the (+)-isomers (Evans, 1980, 1981, 1984a,b, 1993; Evans *et al.*, 1988). The rank order of potency of both the (+)- and (−)-enantiomers of the positional isomers of octopamine (*o*-octopamine = *p*-octopamine > *m*-octopamine) is also different from that found for locust octopamine receptors (Evans *et al.*, 1988) and for vertebrate α- and β-adrenergic receptors (Jordan *et al.*, 1987; Brown *et al.*, 1988). The finding of a [^{3}H]yohimbine binding site in a membrane preparation from *Drosophila* heads with a similar pharmacology to that of the cloned receptor suggests that the unusual pharmacological properties of the latter receptor are not due to its expression in a vertebrate cell line.

The results of the present study reopen the debate as to whether this cloned *Drosophila* aminergic receptor is really

an octopamine (Arakawa *et al.*, 1990) or a tyramine (Saudou *et al.*, 1990) receptor, but the resolution of this question must await the demonstration of its endogenous ligand at specific cellular locations. However, it could be a multifunctional receptor that is activated by octopamine at some locations and by tyramine at other locations, depending upon the identity of the amine released presynaptically. Equally, the receptor may be involved in a novel form of modulation, whereby agents that can alter the ratio of octopamine and tyramine (the immediate biochemical precursor of octopamine) released from octopaminergic neurones in insects, could bias the postsynaptic responses of an effector cell to favour one second messenger system over another.

The cloned *Drosophila* octopamine/tyramine receptor is one of a growing list of receptors that can couple to more than one second messenger system [see Introduction for other examples and Milligan (1993) for a recent review], indicating that this phenomenon is shared by certain groups of both vertebrate and invertebrate G-protein-coupled receptors. The majority of cases reported to date have been found in studies on cloned receptors expressed in cell lines, and the criticism has been raised that the results from such studies may be a function of the overexpression of the receptor in the cell line and not necessarily an indication of its physiological action in its normal environment (Taylor, 1990). However, an increasing number of observations of the same phenomenon are now being reported for endogenously expressed G-protein-coupled receptors (Chakraborty *et al.*, 1991; Crawford *et al.*, 1992; Wolsing and Rosenbaum, 1993). In the present study, the *Drosophila* octopamine/tyramine receptor inhibition of forskolin-stimulated increases in cyclic AMP levels is mediated by a pertussis toxin-sensitive G-protein-coupled pathway, whilst its elevation in intracellular Ca^{2+} levels is mediated via an independent pathway which is pertussis toxin insensitive. It remains to be demonstrated if the receptor can be coupled to both second messenger pathways in an individual *Drosophila* neurone or whether the observed phenomenon reflects the ability of this receptor to couple to different second messenger systems when expressed in different cells with different complements of G-proteins. Further, it seems possible that factors that control the expression of G-proteins themselves could switch the second messenger responses induced by this receptor in a single cell.

An additional level of complexity in the coupling of the cloned *Drosophila* octopamine/tyramine receptor to the two second messenger pathways has been revealed in the present study; namely, that the different agonists tested, octopamine and tyramine, which differ structurally by only the presence of a hydroxyl group on the β-carbon of the octopamine side chain, can differentially couple the receptor to the two second messenger systems studied. In both the ligand-binding studies and cyclic AMP level attenuation studies, tyramine is almost two orders of magnitude more potent than octopamine. In contrast, in the studies on the initiation of the transient intracellular Ca^{2+} signal the dose–response curves for the two amines overlap when the magnitudes of their peak Ca^{2+} signals are compared and octopamine even appears more potent than tyramine when the kinetics of the Ca^{2+} responses are compared. These results are unlikely to be due to genetic drift or cell–cell heterogeneity in our cultures since the effects on cyclic AMP levels and calcium transients have been observed in the same populations of cells at many different passage numbers. Equally, they are unlikely to be due to the production of multiple receptors due to alternative splicing since our binding studies indicate a single binding site for our ligand and since a polymerase chain reaction (PCR) analysis of transfected CHO cell mRNA using closely spaced overlapping primer pairs gave no evidence for the production of multiple transcripts (data not shown). In general terms, agonists differing by only a single hydroxyl in their side chain may be able to bias the interactions of a single G-protein-coupled receptor with multiple second messenger systems by inducing specific conformational changes which enable it to couple preferentially to separate G-proteins. Thus, a single receptor may have a different pharmacological profile depending on which second messenger system is used to assay its efficacy.

Agonist-specific coupling of G-protein-linked receptors to multiple second messenger systems may be a general property shared by a number of different G-protein-linked receptors. Whilst the present paper was in preparation, a cloned type 1 pituitary adenylyl cyclase-activating polypeptide (PACAP) receptor, and four spliced variant forms of the receptor, were reported to be differentially coupled to adenylate cyclase and phospholipase C by two naturally occurring forms of PACAP, namely PACAP-27 and PACAP-38 (Spengler *et al.*, 1993). However, since the cloned *Drosophila* octopamine/tyramine receptor can be differentially activated by agonists differing by only a single hydroxyl group, it has the advantage that site-directed mutagenesis can be used to identify the key amino acid which interacts with this hydroxyl group. This single interaction is likely to be responsible for the initiation of the conformational change in the receptor that leads to the switching of its coupling to the second messenger systems.

Materials and methods

Ligand-binding studies

The transfected CHO-K1 cells were maintained in culture and cell membrane preparations made as described previously (Arakawa *et al.*, 1990). For the radioligand binding assays, aliquots of homogenate (equivalent to 20 μg protein) were incubated at 37°C for 20 min in the presence of 4 nM [methyl-^{3}H]yohimbine (81–89 Ci/mmol; Amersham International), binding assay buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 12 mM EDTA] and in the presence or absence of the appropriate non-labelled competing ligand. Non-specific binding was determined in the presence of 50 μM phentolamine. The final incubation volume was 250 μl. The reaction was started by adding 20 μg membrane protein to the reaction mix and terminated by vacuum filtration over wet Whatman GF/C glass fibre filters. Filters were rinsed with 5 ml of ice-cold buffer and added to 3 ml of scintillant (Ecoscint A) prior to counting. The inhibition constant (K_i) for each ligand was calculated using the EBDA programme of G.A.McPherson, distributed by Elsevier-Biosoft, Cambridge, UK. The mean K_i for each ligand ($\pm$SEM) is shown and the numbers in parentheses represent the number of independent experiments with each point performed in triplicate. The [^{3}H]yohimbine binding showed a K_d of 11.2 $\pm$ 1.7 nM, n = 7 and a B_{max} of 1.8 $\pm$ 0.5 pmol receptor/mg membrane protein by Scatchard analysis (not shown). Hill plots of the data (not shown) gave a coefficient close to 1 (0.91 $\pm$ 0.05, n = 7), indicating a single binding site for the ligand in the CHO cell membranes. No specific radioligand binding was observed to membranes prepared from control, untransfected CHO-K1 cells (Arakawa *et al.*, 1990).

Drosophila head membranes were prepared as described previously (Jackson *et al.*, 1984). Assay conditions were the same as described above except that 50 mM Tris was replaced by 50 mM sodium potassium phosphate, 0.5% sodium metabisulfite was added to assay solutions to stabilize various ligands, 57.6–104.5 μg of membrane protein were used in each assay reaction and the incubation was for 30 min at 30°C, or in some cases for 3 h at 5°C. There was no difference between the two assay temperatures when sodium metabisulfite was used. Filtration generally used GF/B glass fibre filters (but there was no appreciable difference with GF/C filters). Twenty independent Scatchard analyses of [^{3}H]yohimbine binding

to *Drosophila* head extracts using 0.5 μM phentolamine to determine background binding were performed, and gave a K_d of 7.39 ± 1.53 nM and a B_{max} of 447 ± 45.9 fmol/mg membrane protein. The K_is for demethylchlordimeform and amitraz were calculated from composite curves. The variation in *n* values shows the range of independent experiments for different ligand concentrations. Each independent repetition was done in triplicate.

Cyclic AMP attenuation studies

Transfected cells (3×10^5 cells/dish) were washed once with phosphate-buffered saline (PBS) and incubated in PBS containing 1 mM 3-isobutyl-1-methylxanthine (Sigma) at 37°C for 20 min. Agonists, at the indicated concentrations, were added to the cultures along with 10 μM forskolin (Sigma), and cultures were incubated at 37°C for an additional 20 min. Incubations were terminated by the addition of 0.5 ml acidified ethanol (60:1 absolute ethanol:concentrated HCl v/v) to each plate. This was combined with two further 0.25 ml acidified ethanol washes of each plate and centrifugation for 5 min at 2000 *g* at room temperature. The supernatant was evaporated to dryness (80°C for 3 h) and the samples taken up in 150 μl of 0.05 mM Tris buffer (pH 7.5) containing 4 mM EDTA and centrifuged as above. Cyclic AMP levels were measured using a commercial cyclic AMP ^{3}H assay kit (Amersham International). Data represent the average ± SEM of at least three separate experiments performed in duplicate. No inhibition of the forskolin stimulation of cyclic AMP levels was obtained with octopamine or tyramine in control, untransfected cells.

Fura-2 imaging studies

Cells were grown on 22 mm diameter glass coverslips to ~80% confluency, washed and then loaded with 1 μM of the calcium-sensitive dye fura-2-acetoxymethylester (Grynkiewicz *et al.*, 1985) for 30 min at room temperature. Ratio-imaging was carried out at 37°C as described previously (Cheek *et al.*, 1989, 1993; Tsien and Harootunian, 1990). Fluorescent images were obtained by alternate excitation at 340 or 380 nm (40 ms each wavelength) using an image-processing system (Imagine, Synoptics, Cambridge) interfaced to a DEC Microvax II microcomputer. The ratio image was obtained at video rate and filtered with a time constant of 200 ms (i.e. 5 ratio images/s). Cells were perfused at 37°C with Ca^{2+}-containing PBS from a main reservoir, and agonist in the same buffer applied via a U-tube positioned to within 2 mm of the field of cells under observation. Experiments with dye solutions showed that by using this method all the cells in the field are exposed virtually simultaneously to the agonist and within 1 s of the onset of application. The lag times and times to peak of the Ca^{2+} transients were measured automatically by the computer which, for the data shown in Figure 2D and E, was set to identify the point where the calcium transient reached 15 nM above the basal level to identify the start of the transient. No calcium transients were observed to the application of either octopamine or tyramine (100 μM) in control, untransfected cells.

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