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Interaction of anandamide with the M_1 and M_4 muscarinic acetylcholine receptors

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Abstract

The M_1 and M_4 muscarinic acetylcholine receptors are the most abundant muscarinic receptor subtypes in the brain, and are involved in learning and memory. Because cannabinoid receptors are also abundantly expressed in similar brain regions and mediate opposite effects to acetylcholine on cognition, the present study investigated whether the endocannabinoid agonist, anandamide, and its metabolically stable derivative, methanandamide, directly modified the binding properties of the human M_1 and M_4 receptors individually expressed in CHO cell membranes. Experiments utilized the antagonists, [^{3}H]*N*-methylscopolamine and [^{3}H]quinuclidinyl benzilate. When acetylcholine was used as the inhibiting ligand, shallow, biphasic isotherms were observed at both receptors, characterised by similar apparent dissociation constants for high and low affinity binding at each receptor but with a greater proportion of high affinity sites at the M_4 (40–45%) than at the M_1 receptor (17–20%). In contrast, anandamide and methanandamide inhibited the binding of both radioligands over a narrow (low micromolar) concentration range, with monophasic isotherms characterized by Hill coefficients significantly greater than 1 at both receptors. These effects were not due to the vehicle used. Further saturation binding analyses found anandamide able to significantly reduce the apparent affinity and maximal density of binding sites labeled by [^{3}H]quinuclidinyl benzilate. Interestingly, no significant inhibition of radioligand binding was noted using the synthetic cannabinoid agonist, WIN55212-2, or the cannabinoid CB_1 receptor antagonist, SR141716A. These data thus provide evidence for a direct role of anandamides in modulating muscarinic receptor binding properties through a non-competitive mechanism that is unrelated to their actions on cannabinoid receptors.

Theme: Neurotransmitters, modulators, transporters, and receptors

Topic: Acetylcholine receptors: muscarinic

Keywords: Acetylcholine; Anandamide; Cannabinoid; Muscarinic receptor; Memory

1. Introduction

Both acetylcholine and cannabinoid ligands have long been known to exert profound effects on a variety of processes associated with cognition, with the former agent facilitating and the latter substances causing disruptions in learning and memory [15–17,21,29]. At the molecular level, the majority of the effects of acetylcholine and cannabinoid ligands are mediated by specific receptors belonging to the G protein-coupled receptor superfamily [4,5,26,27].

In terms of effects on memory, much attention has focused on muscarinic and cannabinoid-induced changes in the release or actions of glutamate, especially due to the known localization of many hippocampal receptors for each of these substances on or near glutamatergic neurones [15,17]. However, cannabinoids are also able to interfere with the synthesis [13] and/or release of acetylcholine [9], and this provides another potential mechanism by which cannabinoids can exert negative modulatory effects on cognition. Indeed, more recent studies have illustrated a physiological antagonism between central cholinergic and cannabinoid mechanisms in their effects on working memory [3,30].

With the relatively recent identification of an endogenous cannabinoid signaling system, exemplified by the arachidonic acid derivative, anandamide (Fig. 1), attention

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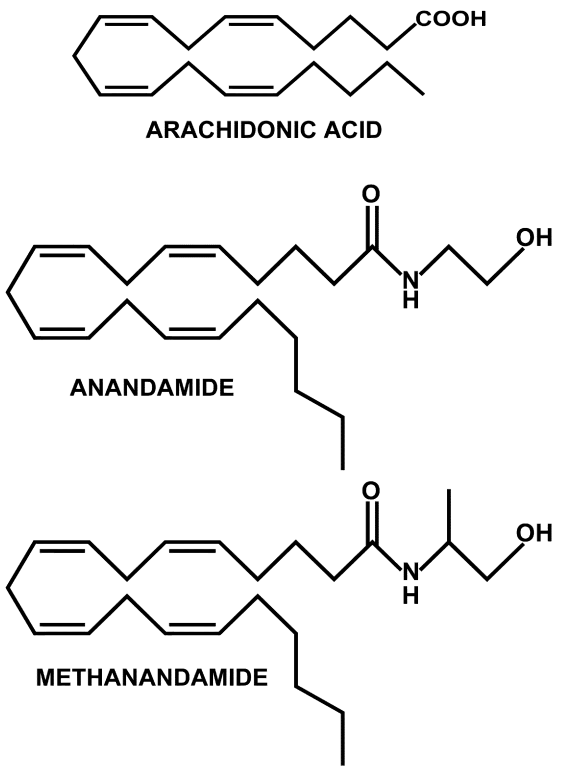

Fig. 1. Structures of arachidonic acid, anandamide and methanandamide.

has now been focused on the possibility of more direct effects of the endocannabinoids on other receptor systems. For example, anandamide, as well as binding with nanomolar affinity to the cannabinoid receptors and acting as an agonist [26], has also been shown to inhibit ligand binding to central 5-HT receptors [19], although no effects were noted on ionotropic $GABA_A$ receptors in the same study. More recently, Frey et al. demonstrated that anandamide was also able to inhibit ligand binding to muscarinic acetylcholine receptors in homogenates derived from human brain [22]. That finding suggests that anandamide may play a more direct role on central cholinergic processes beyond its effects on cannabinoid receptors. However, the human brain is known to contain a mixture of all five subtypes of muscarinic receptors, particularly the M_1 and M_4 [23,31], and the effects of anandamide on the binding properties of the individual human muscarinic receptors are currently unknown. Because M_1 and M_4 receptors, as well as the cannabinoid CB_1 receptors, are richly expressed in brain regions associated with learning and memory, the present study was undertaken in order to elucidate the effects of anandamide, and its metabolically stable derivative methanandamide (Fig. 1), on the individual binding properties of these two muscarinic receptor subtypes using CHO cell membranes stably expressing the human M_1 or M_4 receptor.

2. Materials and methods

2.1. Materials

[^{3}H]*N*-methylscopolamine (70 Ci/mmol) and [^{3}H]quinuclidinyl benzilate (43 Ci/mmol) were purchased from NEN Dupont (Wilmington, DE); Dulbecco's modified Eagle's medium was purchased from Gibco BRL (Gaithersburg, MD); geneticin was obtained from Calbiochem (La Jolla, CA); bovine calf serum was supplied by Hyclone (Logan, UT); WIN 55,212-2 (*R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]-pyrrolo[1,2,3,-de]-1,4-benzoxazin-yl]-(1-napthalenyl)-methanone mesylate) was purchased from Research Biochemicals Incorporated (Natick, USA) and SR141716A (*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide) was a generous gift from Sanofi-Recherche, Montpellier, France. All other reagents were purchased from Sigma Chemical Co.

2.2. Cell culture

Chinese hamster ovary (CHO) cells, stably expressing the human M_1 or M_4 muscarinic acetylcholine receptor (provided by Dr. M. Brann, University of Vermont Medical School, Burlington, VT) were grown for 4 days at 37°C in Dulbecco's modified Eagle's medium, supplemented with 10% bovine calf serum and 50 μg/ml geneticin, in a humidified atmosphere consisting of 5% CO_2 and 95% air. Cells were harvested 4 days after subculture by trypsinization followed by centrifugation (300×*g*, 3 min) and resuspension of the pellet in HEPES buffer (0.03 M HEPES, 0.5 mM $MgCl_2$, 0.5 mM EGTA; pH 7.4), repeated twice.

2.3. Membrane preparation

CHO cells were harvested as described above and centrifuged (300×*g*, 3 min) before re-suspension of the pellet in HEPES buffer. The pellet was washed twice and resuspended in HEPES buffer prior to homogenization with a Polytron homogenizer (2×10 s bursts, with a 30 s period of cooling on ice between homogenizations) and centrifugation at 1000×*g* for 10 min. The resulting supernatant was collected and centrifuged at 30,000×*g* for 30 min, after which the pellet (P_2 fraction) was re-suspended and immediately utilized in the binding assays. Protein content was determined by the method of Bradford [2], with bovine serum albumin as the standard.

2.4. Inhibition binding assays

CHO cell membranes expressing either the M_1 receptor (4 μg/tube) or the M_4 receptor (2 μg/tube) were incubated with 50 pM of the lipophilic antagonist, [^{3}H]quinuclidinyl benzilate, or 0.2 nM of the hydrophilic

antagonist, [^{3}H]*N*-methylscopolamine, in the absence or presence of increasing concentrations of acetylcholine, anandamide or methanandamide in a total volume of 1 ml. Subsequent experiments also investigated the interaction between [^{3}H]quinuclidinyl benzilate and the cannabinoid agonist, WIN 55,212-2 or antagonist, SR141716A. Anandamide and methanandamide were prepared as 10 mM stock solutions in ethanol, while WIN55,212-2 and SR141716A were prepared as 10 mM stock solutions in dimethylsulfoxide. Subsequent dilutions were made in HEPES buffer. Incubation was allowed to proceed for 1 h at 37°C before termination by rapid filtration through Whatman GF/B filters, positioned on a Brandell Cell Harvester. Filters were washed three times with 4-ml aliquots of ice-cold saline and dried before radioactivity was measured using liquid scintillation counting. Non-specific binding was defined using 10 μM atropine.

2.5. Saturation binding assays

CHO cell membranes expressing either the M_1 receptor (4 μg/tube) or the M_4 receptor (2 μg/tube) were incubated with increasing concentrations of [^{3}H]quinuclidinyl benzilate or [^{3}H]*N*-methylscopolamine in a total volume of 1 ml for 1 h at 37°C before termination and determination of radioactivity as described above for the inhibition experiments. Additional [^{3}H]quinuclidinyl benzilate saturation experiments were also conducted in parallel in the presence of a fixed concentration of anandamide (see Results). In all instances, non-specific binding was defined using 10 μM atropine.

2.6. Data analysis

Inhibition binding isotherms were analyzed via non-linear regression using PRISM 3.02 (GraphPad Software, San Diego, CA) in order to derive estimates of the Hill slope factor and the IC_{50} (midpoint location or potency parameter). Assuming simple competition, the data were re-fitted according to both one- and two-site mass-action binding models and the better model was determined by an extra-sum-of-squares test using PRISM. IC_{50} values were converted to K_I values (competitor-receptor dissociation equilibrium constant) according to the following equation [6]:

$$K_I = \frac{IC_{50}}{1 + [D]/K_D}$$

where $[D]$ and K_D denote the concentration and dissociation constant of the radioligand, respectively. In instances where the data did not conform to a simple mass action competitive model, pIC_{50} values only are quoted in the text as empirical estimates of inhibitor potency.

Results are expressed as mean±standard error of the mean. Statistical significance was determined by paired or unpaired *t*-test, or by one-way analysis of variance (ANOVA), as appropriate. Unless otherwise stated, a probability (P) value of 0.05 was taken to indicate statistical significance.

3. Results

3.1. Binding of acetylcholine to the M_1 and M_4 receptors

Table 1 shows the saturation binding parameters for both radioligands at the M_1 and M_4 muscarinic acetylcholine receptors. Although there was a trend towards a higher affinity for [^{3}H]quinuclidinyl benzilate relative to [^{3}H]*N*-methylscopolamine, a one-way ANOVA found no significant difference between any of the parameters at the M_1 and M_4 receptors.

Subsequent competition experiments were undertaken to characterize the binding of the endogenous muscarinic receptor agonist, acetylcholine, to the CHO M_1 and M_4 receptors (Fig. 2). Irrespective of the radioligand used, acetylcholine was able to completely inhibit specific binding in a similar fashion, yielding shallow competition isotherms with Hill slopes significantly less than unity (Table 2). Nonlinear regression analysis of the data revealed the competition to be best described in terms of two distinct affinity states for the agonist (Table 2). Overall, the agonist binding parameters were not significantly different between receptor subtypes or radioligands used, except for the proportion of high affinity states recognised, which were significantly greater for the M_4 receptor (Table 2).

3.2. Binding of anandamides to the M_1 and M_4 receptors

In contrast to the shallow competition curves observed

Table 1
Saturation binding parameters for [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) or [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) at the human M_1 or M_4 muscarinic acetylcholine receptors expressed in CHO cell membranes. Values are the mean±S.E.M. of experiments conducted in duplicate

Parameter	M_1		M_4	
	[^{3}H]NMS	[^{3}H]QNB	[^{3}H]NMS	[^{3}H]QNB
B_{max} [a]	5.15±0.26	6.84±1.24	5.79±1.23	5.89±1.04
pK_D [b]	10.32±0.19	10.61±0.12	10.15±0.04	10.46±0.08
n [c]	8	4	6	4

[a] Maximal density of binding sites, expressed as pmol/mg protein.
[b] Negative logarithm of the radioligand dissociation constant.
[c] Number of experiments.

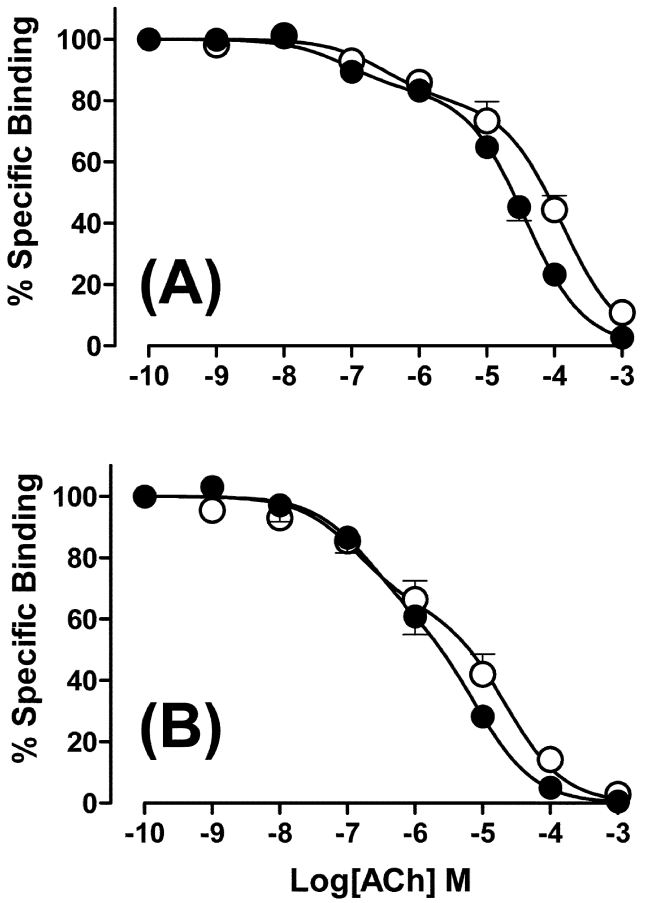

Fig. 2. Inhibition binding curves for acetylcholine at (A) the M_1 muscarinic acetylcholine receptor or (B) M_4 muscarinic acetylcholine receptor using 0.2 nM [^{3}H]*N*-methylscopolamine (●) or 50 pM [^{3}H]quinuclidinyl benzilate (○) as the radioligands. Incubation was for 1 h at 37°C. Non-specific binding was defined using 10 μM atropine. Points represent the mean±S.E.M. of four experiments conducted in duplicate. Where error bars are not shown, they lie within the dimensions of the symbol.

Table 2
Inhibition binding parameters for acetylcholine against [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) or [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) at the human M_1 or M_4 muscarinic acetylcholine receptors expressed in CHO cell membranes. Values are the mean±S.E.M. of four experiments conducted in duplicate

Parameter	M_1		M_4	
	[^{3}H]NMS	[^{3}H]QNB	[^{3}H]NMS	[^{3}H]QNB
pK_H[a]	7.41±0.32	7.05±0.38	7.02±0.27	7.33±0.25
pK_L[b]	4.78±0.06	4.37±0.09	5.42±0.17	5.13±0.16
% Hi[c]	17±3	20±4	40±10	45±7
n_H[d]	0.62±0.06	0.56±0.05	0.56±0.06	0.50±0.04

[a] Negative logarithm of the dissociation constant for the high-affinity agonist binding site.
[b] Negative logarithm of the dissociation constant for the low-affinity agonist binding site.
[c] Percentage of high affinity binding sites.
[d] Hill slope factor.

with acetylcholine, both anandamide and methanandamide were able to completely inhibit radioligand binding over a very narrow concentration range, yielding isotherms with Hill slopes significantly greater than unity (Figs. 3 and 4; Table 3). This effect was not an artefact related to the physicochemical properties of the radioligands, as the binding profile was similar whether the lipophilic [^{3}H]quinuclidinyl benzilate (Fig. 3) or the hydrophilic [^{3}H]*N*-methylscopolamine (Fig. 4) was used. Because the antagonism demonstrated by the anandamides deviated from simple mass action kinetics, Table 3 lists only the pIC_{50} values for both compounds in inhibiting radioligand binding. Nevertheless, these values are useful as empirical estimates of inhibitor potency under similar assay conditions and illustrate that significant effects on the binding of the radioligands to either the M_1 or M_4 receptor occur

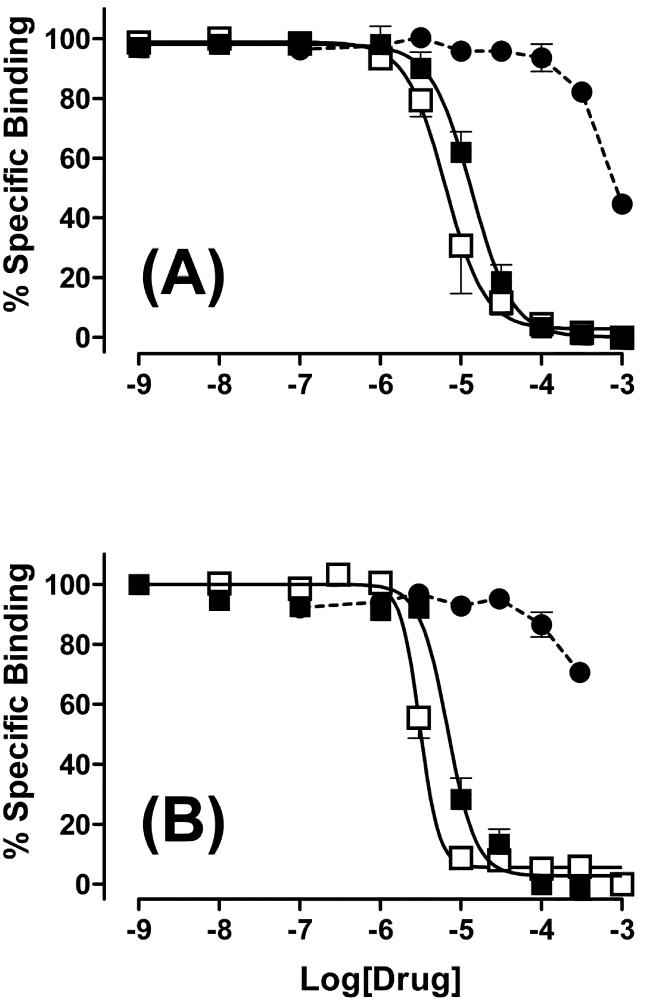

Fig. 3. Inhibition binding parameters for anandamide (■), methanandamide (□) or ethanol vehicle equivalents (●) against 50 pM [^{3}H]quinuclidinyl benzilate at the M_1 (A) or M_4 (B) muscarinic acetylcholine receptor expressed in CHO cell membranes. Data represent the mean±S.E.M. of five experiments conducted in duplicate. All other details as for Fig. 2.

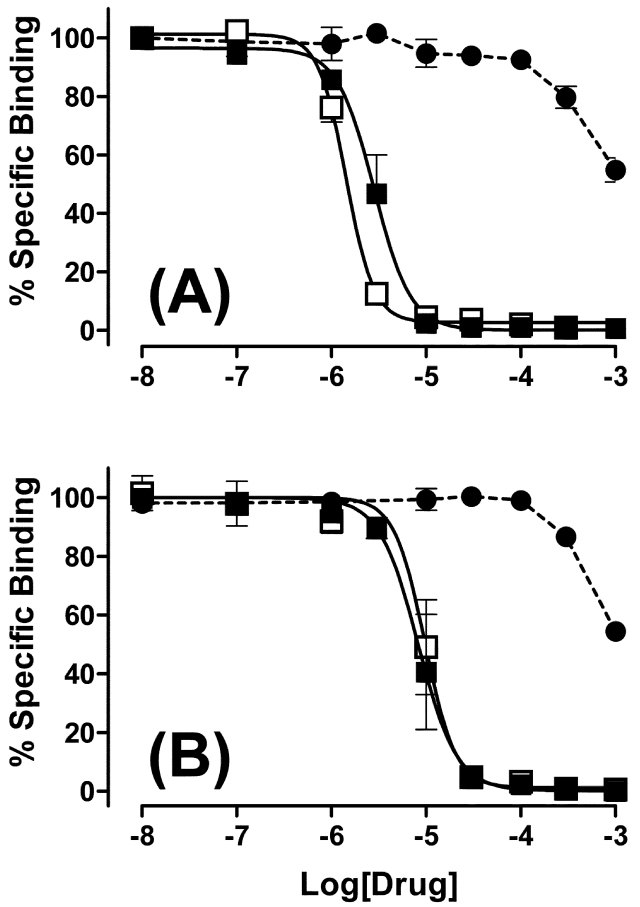

Fig. 4. Inhibition binding parameters for anandamide (■), methanandamide (□) or ethanol vehicle equivalents (●) against 0.2 nM [^{3}H]*N*-methylscopolamine at the M_1 (A) or M_4 (B) muscarinic acetylcholine receptor expressed in CHO cell membranes. Data represent the mean±S.E.M. of five experiments conducted in duplicate. All other details as for Fig. 2.

in the low-to-mid micromolar inhibitor concentration range (Table 3).

Figs. 3 and 4 also illustrate the effects of the ethanol vehicle concentration equivalents on radioligand binding, where it can be seen that no significant effects were noted for the vehicle over the concentration ranges where the anandamides completely inhibited binding. Additional experiments, using incubation times of 90 min, also found no significant change in the binding profile of the anandamides at either receptor type (data not shown).

Table 3
Inhibition binding parameters for anandamide or methanandamide against [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) or [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) at the human M_1 or M_4 muscarinic acetylcholine receptors expressed in CHO cell membranes. Values are the mean±S.E.M. of five experiments conducted in duplicate

Parameter	M_1		M_4	
	[^{3}H]NMS	[^{3}H]QNB	[^{3}H]NMS	[^{3}H]QNB
Anandamide				
pIC_{50} [a]	5.55±0.04	4.87±0.04	5.08±0.06	5.16±0.04
n_H [b]	2.23±0.42	1.72±0.24	2.13±0.67	2.48±0.06
Methanandamide				
pIC_{50}	5.84±0.02	5.19±0.04	5.01±0.04	5.51±0.02
n_H	2.98±0.34	1.77±0.36	2.69±0.41	2.67±0.04

[a] Negative logarithm of the inhibitor concentration that reduces specific radioligand binding by 50%.
[b] Hill slope factor.

3.3. Binding of cannabinoids to the M_1 and M_4 receptors

In order to ascertain whether the effects of the anandamides were related to the cannabinomimetic nature of these compounds, further experiments were undertaken using [^{3}H]quinuclidinyl benzilate in competition with the cannabinoid agonist, WIN 55,212-2, or antagonist, SR141716A. As shown in Fig. 5, neither agent signifi-

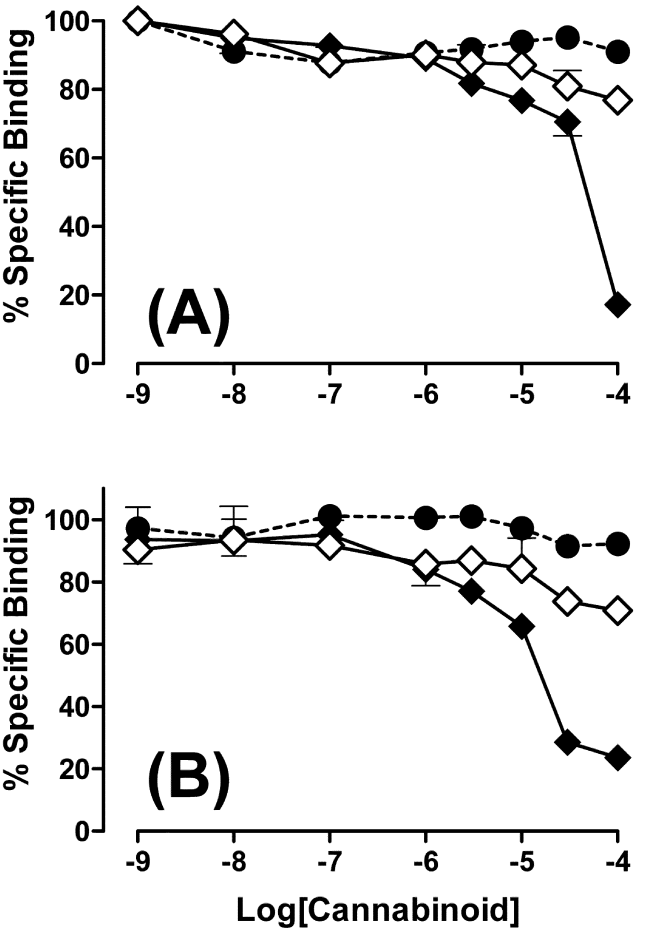

Fig. 5. Inhibition binding parameters for WIN 55,212-2 (◇), SR141716A (◆) or dimethylsulfoxide vehicle equivalents (●) against 50 pM [^{3}H]quinuclidinyl benzilate at the M_1 (A) or M_4 (B) muscarinic acetylcholine receptor expressed in CHO cell membranes. Data represent the mean±S.E.M. of three experiments conducted in duplicate. All other details as for Fig. 2.

cantly affected radioligand binding, except for the highest concentration of SR141716A.

3.4. Effect of anandamide on [^{3}H]quinuclidinyl benzilate saturation binding

Since steep Hill slopes in inhibition binding assays may be indicative of a number of non-competitive ligand–receptor interactions, additional [^{3}H]quinuclidinyl benzilate saturation binding assays were undertaken in order to gain further insight into possible mechanisms underlying the anandamide effects. Fig. 6 illustrates the results of these experiments, where it can be seen that the presence of an IC_{50} concentration of anandamide (as determined in the inhibition experiments above) altered both the location and the maximal density of binding sites recognized by the radioligand at the M_1 (Fig. 6A) and M_4 (Fig. 6B) receptors. These dual effects of anandamide are illustrated more clearly in Fig. 6C, for the radioligand pK_D values, and Fig. 6D for the B_{max} values. In the presence of anandamide, the actual parametric values were as follows: M_1, $pK_D = 10.07 \pm 0.06$, $B_{max} = 5.36 \pm 1.24$ pmol/mg; M_4, $pK_D = 9.68 \pm 0.06$, $B_{max} = 2.87 \pm 1.24$ pmol/mg. In the absence of anandamide, the values were as shown in Table 1.

4. Discussion

In the present study, we have identified a direct effect of the endocannabinoid, anandamide, on the binding properties of the human M_1 and M_4 muscarinic acetylcholine receptors. Importantly, these proteins are the most abundantly expressed subtypes of muscarinic acetylcholine receptors in the hippocampus [23,31], a region that has a well-established role in memory processing. Our findings suggest a potentially novel mode of regulation of central cholinergic processes by endogenous lipid neuromodulators.

The finding that anandamide and its metabolically stable derivative, methanandamide, completely inhibit

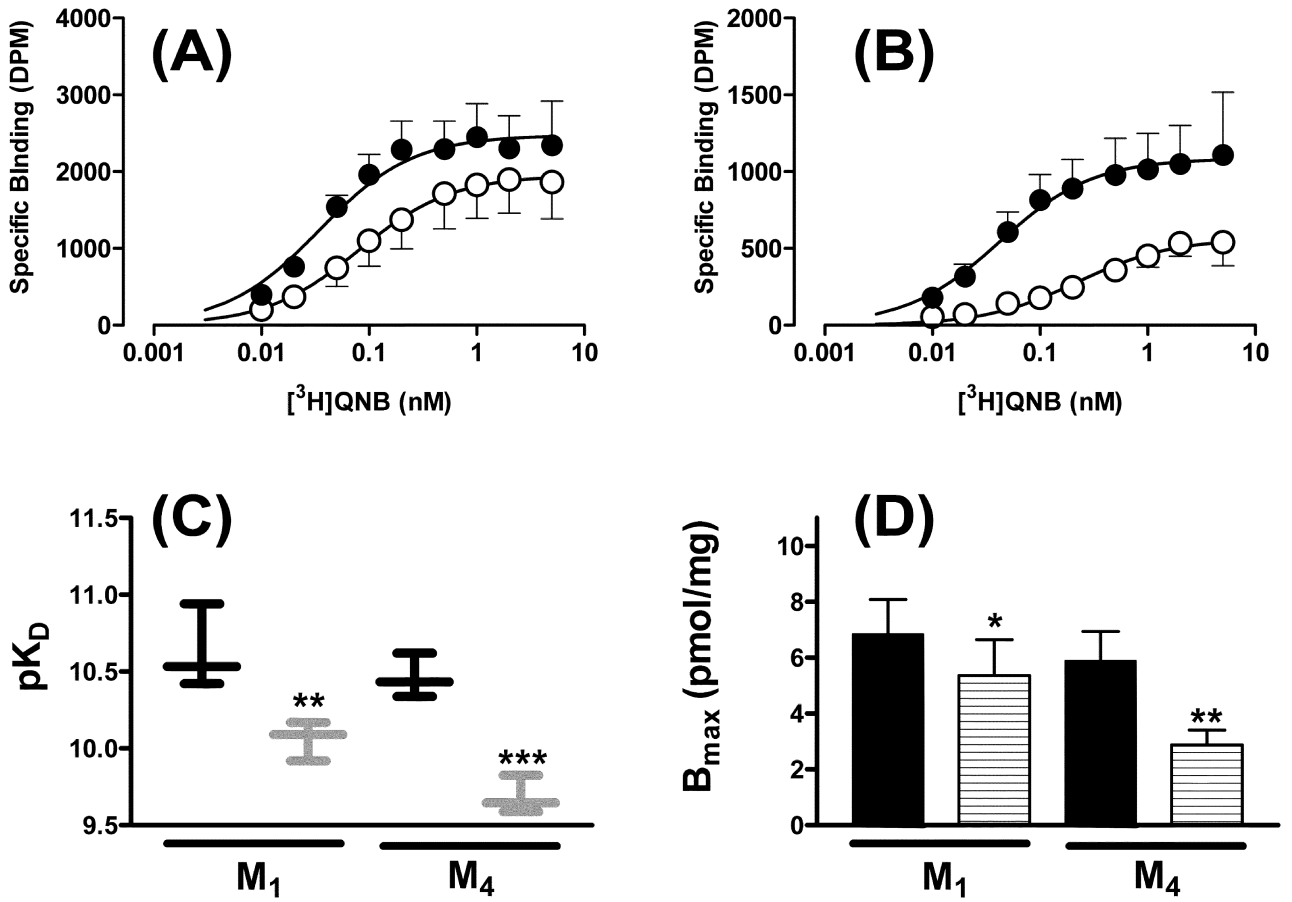

Fig. 6. Saturation binding of [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) in the absence (●) or presence (○) of (A) 3 μM anandamide at the M_1 muscarinic acetylcholine receptor or (B) 8 μM anandamide at the M_4 muscarinic acetylcholine receptor. Incubation was for 1 h at 37°C and non-specific binding was defined using 10 μM atropine. Also shown are (C) the effects of anandamide (light bars) on the radioligand pK_D value relative to control (dark bars) and (D) the effects of anandamide (hatched bars) on the radioligand B_{max} value relative to control (dark bars). Data represent the mean±S.E.M. of 4–5 experiments conducted in duplicate. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

radioligand binding to the cloned human muscarinic acetylcholine receptors confirms a previous report that used homogenates derived from adult human frontal cortex [22]. However, the cortex possesses a mixture of all five subtypes of muscarinic acetylcholine receptor [4,5,12], and thus the effects of the anandamides on individual muscarinic subtypes could not be ascertained in that study. Our findings have established that the anandamides are able to interact with both the M_1 and M_4 muscarinic receptors.

There are a number of important mechanistic aspects related to the binding of the anandamides observed in this study. For example, the inhibition of radioligand binding by the anandamides was characterized by extremely steep isotherms with Hill slopes significantly greater than unity (Figs. 3 and 4; Table 3). This is in marked contrast to the inhibition produced by acetylcholine, which was shallow and characterized by multiple affinity states (Fig. 2; Table 2). In the latter instance, biphasic competition binding isotherms are quite characteristic for many G protein-coupled receptors [18]; the deviation from simple mass-action in this case tends to reflect the distribution of agonist-bound receptor amongst G protein coupled and uncoupled forms [8]. Indeed, the significantly greater percentage of high affinity binding sites for acetylcholine at the M_4 relative to the M_1 receptor noted in this study probably reflects differences in the efficiency and/or the stoichiometry of receptor-G protein coupling between the two receptor types. However, the steep inhibition binding curves noted with the anandamides are not in accord with the predictions of the simple ternary complex model of agonist-receptor-G protein action, and alternative mechanisms need to be considered.

It may be argued that our findings with the anandamides reflect experimental artefacts, such as inhibitor solubility problems or inadequate ligand equilibration times; both mechanisms can contribute to steep ligand binding curves. However, this is unlikely to be the case due to reasons outlined below. The highly lipid nature of the anandamides necessitates the use of vehicles such as ethanol or dimethylsulfoxide. Although we can conclude that the vehicle used did not significantly contribute to the inhibition of radioligand binding observed with the anandamides (Figs. 3 and 4), it is possible that the sequential dilution of the vehicle as a consequence of the serial dilutions of the anandamide stock solutions resulted in the lowest concentrations of anandamide coming out of solution. As a consequence, this would have the effect of 'steepening' the inhibition curve due to a lack of activity of the lowest inhibitor concentrations. However, additional experiments that maintained a constant and relatively high percentage (5%) of ethanol vehicle for all anandamide concentrations found no change in the resulting binding profile (data not shown). Similarly, increasing the radioligand and inhibitor equilibration times from 60 to 90 min also caused no change in the observed binding profile, thus arguing against an inadequate contact time for the low inhibitor concentrations as another potential cause of the steep inhibition curves.

Thus, the mechanism of anandamide binding to the muscarinic M_1 and M_4 receptors may involve a true non-competitive interaction, such as co-operative binding, pseudo-irreversible binding or membrane-mediated receptor conformational changes. The [^{3}H]quinuclidinyl benzilate saturation experiments shown in Fig. 6 were undertaken in order to provide some insight into these mechanisms. The effect of an IC_{50} concentration of anandamide on radioligand occupancy was characterized by both a rightward shift in the saturation curve and a reduction in the maximal density of receptors recognized by the radioligand. Taken together, these dual effects can account for the steep isotherms noted in the inhibition binding assays. Although a simple irreversible binding mechanism utilizing the classic binding site on the muscarinic receptor would be consistent with reductions in B_{max}, it cannot account for a concomitant change in apparent radioligand affinity. For this to occur, more complex reaction schemes, perhaps cooperative binding in oligomeric receptor arrays [32], would need to be invoked. Certainly, overexpression of receptors in recombinant expression systems, such as CHO cell lines, may predispose the receptors towards oligomer formation; expression levels in our CHO cells were close to 6 pmol/mg protein (Table 1) whereas previous estimates of muscarinic receptor density in rat and human brain have been in the order of 1–3 pmol/mg protein (e.g. Refs. [10,22]). Nevertheless, effects of anandamide on muscarinic acetylcholine receptor binding properties similar to those reported herein have been observed in human brain tissue [22], and cooperative interactions have been documented for muscarinic acetylcholine receptors in other native tissues as well [7].

It should be noted that effects of eicosanoids, such as anandamide and arachidonic acid, have also been noted on the binding properties of other receptors. For instance, Kjome et al. [20] demonstrated similar effects of arachidonic acid on human brain muscarinic receptors, while finding nicotinic receptor binding properties relatively unaffected. However, it is unlikely that the effects of anandamide observed in our present study were in fact due the properties of its breakdown to arachidonic acid, because CHO cells do not appear to possess an anandamide removal mechanism [14]. Furthermore, the metabolically more stable methanandamide also yielded almost identical results to those obtained with anandamide. In another study, Kimura et al. [19] have shown anandamide to inhibit the binding of both [^{3}H]5-HT and [^{3}H]ketanserin to bovine brain 5-HT receptors while finding no effect of anandamide on the binding properties of the $GABA_A$ ionophore receptor complex. A further examination of the 5-HT receptor binding data reported by Kimura et al. [19] also reveals an important difference between their findings and ours; whereas we noted steep anandamide inhibition curves, their data described shallow

binding isotherms (see Tables 3 and 4 in Ref. [19]). Although we cannot rule out a general membrane perturbation effect of the eicosanoids as the underlying cause of their effects on binding, the observations described above point to a degree of specificity of the anandamide effect for G protein-coupled as opposed to ion channel-linked receptors, as well as differences in the nature of its manifestation across different G protein-coupled receptor types.

Besides the mechanistic considerations outlined in the preceding paragraphs, a second important aspect of the effects of the anandamides relates to their structure–activity relationships. Although the synthetic cannabinoids WIN 55,212-2 and SR141716A both bind to brain cannabinoid receptors with much higher affinity than anandamide and methanandamide [26], they failed to exert significant effects on muscarinic receptor binding properties over similar concentration ranges (Fig. 5). Thus, it is unlikely that the direct effects of the anandamides on the M_1 and M_4 receptors are related to their cannabinomimetic properties, but rather to their eicosanoid nature, as arachidonic acid is also able to affect muscarinic receptor binding [20].

Perhaps the most important issue related to the interaction between the anandamides and the muscarinic acetylcholine receptors is that of physiological (or pathophysiological) relevance in terms of both functional endpoints and the levels of endogenous anandamide that would be required to observe such an interaction. To date, there have been no reports of a functional interaction between anandamide and muscarinic receptors, although the study of Lagalwar et al. [22] did demonstrate an effect of anandamide on the binding properties of the agonist, oxotremorine-M. Preliminary experiments in our laboratory suggest that anandamide can modify muscarinic acetylcholine receptor signaling properties in a ligand-specific manner (Lanzafame and Christopoulos, unpublished observations). With regards to the endogenous production of anandamide, the hippocampus and cortex produce amongst the highest levels of anandamide in the brain [1,11], however absolute basal levels of this substance have been reported to be very low, at least 10-fold lower than other classic neurotransmitters [25]. Since its actions on the cannabinoid receptors occur in the nanomolar to submicromolar range, it may be argued that anandamide cannot exert significant effects directly on muscarinic receptor binding under normal conditions, because this requires at least low to mid micromolar concentrations (Table 3). However, a number of points need to be raised regarding this matter. Firstly, accurate determinations of anandamide levels have generally been hampered to date due to the extraordinarily rapid inactivation mechanisms associated with its release [28]. Indeed, it is still unclear what levels of local anandamide concentrations can actually be achieved under stimulated conditions, although one study has suggested 0.1 μM under non-stimulated conditions [33]. Secondly, there are disorders associated with enhanced anandamide production, including schizophrenia [24] and ischaemia [11], thus suggesting at least two pathophysiological states that can cause significant elevations in local anandamide concentrations. Finally, the other well-characterized endocannabinoid, 2-arachidonyl glycerol, is actually present in considerably greater amounts than anandamide (at least 200-fold) in all tissues thus far known to produce them; the possibility that 2-arachidonyl glycerol itself can also exert effects on other receptor systems is thus also worthy of consideration.

In conclusion, we have identified a direct effect of the endocannabinoid, anandamide, and its metabolically stable derivative, methanandamide, on the human M_1 and M_4 muscarinic acetylcholine receptors. This effect appears to be noncompetitive in nature, and likely related to the eicosanoid structure of anandamide, rather than its cannabinomimetic properties. The former mechanism may be particularly relevant under conditions of elevated anandamide levels as seen, for instance, in schizophrenia or various ischaemic states.

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