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MULTIPLE CENTRAL α_2 ADRENOCEPTORS OF AVIAN AND MAMMALIAN SPECIES

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Abstract—Although equilibrium binding experiments indicated that calf cerebral membranes contained two classes of clonidine receptors and that chicken cerebral membranes might have contained only one, experiments investigating the kinetics of binding and the effects of GppNHp clearly indicated that the cerebral membranes of both species contained two subtypes of receptor, with the avian high affinity receptor having been present at too low a density to be readily detected in equilibrium binding studies. For both species 10 μM GppNHp sharply reduced or eliminated both the high affinity binding site and the slow steps of association and dissociation without changing the low affinity site and its related rapid association and dissociation steps. The high affinity sites from both species had similar specificities since the relative affinities of the avian binding site for a series of clonidine analogues closely reflected the relative affinities of the calf binding site. The properties of the chicken and calf α_2 subtypes resembled those reported for rat brain.

Recent radioligand binding investigations suggest that central α_1 and α_2 adrenoceptor subtypes are heterogenous. It has been demonstrated, for instance, that the α_1 adrenoceptor, as defined by the binding of the labeled alpha antagonist [^{3}H]WB-4101, is composed of two subsites with differential affinity for the radioligand in cerebral cortical membrane suspensions (CMS) from calf [1] and mouse [2].

Similarly, it has been reported that the α_2 adrenoceptor, as determined by the binding of [^{3}H]clonidine to cerebral homogenates, is composed of high and low affinity subtypes. This differentiation has not been observed by all investigators, however, since studies of [^{3}H]clonidine binding to rat brain have been interpreted in terms of a single binding site by some [3–7] but in terms of two binding sites by others [8–10].

The interaction of clonidine with avian cerebral membranes was of considerable interest since clonidine has marked antifertility action in laying hens [11]. Accordingly, we initiated a study investigating the binding of clonidine to avian (laying hen) cerebral membranes. In preliminary equilibrium binding studies, the binding of clonidine to avian membranes appeared monophasic, while earlier results from this laboratory [1] and others [12] indicated that its binding to bovine membranes was clearly biphasic. This suggested that there might be a phylogenetic difference in the number of α_2 receptor subtypes. To investigate this possibility, we have examined in detail the kinetics of binding and the equilibrium binding of clonidine to avian and bovine cortical membranes. We have also investigated the effects of the nonhydrolyzable nucleotide GppNHp, since guanyl nucleotides specifically affect the high affinity clonidine binding site in rat brain homogenates [10]. Finally, the affinities of a series of clonidine analogues at calf and chicken cerebral membrane receptors have been compared. The results of these experiments are the subject of this paper.

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MATERIALS AND METHODS

Receptor binding assay. [^{3}H]Clonidine (2-[2,6-dichlorophenyl-[4-^{3}H]amino]-2-imidazoline hydrochloride) was obtained from the New England Nuclear Corp., Boston, MA, at a specific activity of 22.2 to 23.8 Ci/mmole, and stored in ethanol–water (7:3) at 0°C. The radiochemical purity of this ligand was checked periodically by thin-layer chromatography on E. Merck silica gel 60F-254 plates [*n*-butanol–acetic acid–water, 4:1:5 (top layer) or benzene–ethanol–dioxan–ammonia [10:8:1:1]. Glass laboratory ware was used. Protein concentrations were determined by a modification of the Lowry procedure [13].

Assays were conducted using homogenates of previously frozen (−75°) chicken or calf cerebral cortex. A few determinations were conducted with fresh chicken cortex to check for artifacts of freezing. A Brinkmann Polytron PT-10 (setting 6, 10 sec) was used to homogenize sections of calf or chicken cerebral cortex in 20 vol. (w/v) of ice-cold 50 mM Tris–HCl buffer, pH 7.7. The resulting homogenate was centrifuged twice at 48,000 *g* (Sorvall SS-34 rotor, 20,000 r.p.m., RC-5 centrifuge) for 10 min at 4°, with rehomogenization of the intermediate pellet in 20 vol. of fresh buffer. The final pellet was resuspended in 50 vol. of ice-cold buffer.

Standard radioligand binding inhibition assays utilized a [^{3}H]clonidine final concentration of 1.0 nM with chicken or 0.2 nM with calf cortical homogenates. Triplicate assay tubes contained [^{3}H]clonidine, 100 μl of various concentrations of the compound being tested, 1000 μl of tissue homogenate, and

50 mM Tris–HCl buffer, pH 7.7, to a final volume of 2000 μl. The reaction was initiated by the addition of tissue, and incubation was continued for 30 min at 25°, at which time it was terminated by rapid filtration through Whatman GF/B glass–fiber filters under vacuum. Each filter was immediately rinsed with 3 × 5 ml aliquots of ice-cold buffer. The filters were removed into 10 ml PCS scintillation fluid (Amersham) and counted on either a Packard model 2425 or 460C scintillation spectrometer at an efficiency of 35%. Specific binding was defined as the difference between samples with and without 1000 nM or 100 nM unlabeled clonidine for assays utilizing chicken or calf cortical homogenates respectively.

Determinations of the binding parameters for the interaction of [^{3}H]clonidine with cerebral cortical membrane preparations from chicken or calf were conducted with concentrations of radioligand ranging from 0.20 to 50 nM (chicken) or 0.01 to 50 nM (calf). Either 1000 nM unlabeled clonidine or 10,000 nM unlabeled (−)-norepinephrine was used to define specific binding.

Assays for the determination of the kinetics of association and dissociation of the radioligand from cortical homogenates employed concentrations of the labeled and unlabeled ligands identical to those employed in standard radioligand binding inhibition assays. For dissociation kinetics determinations, the final pellet of cerebral cortex was resuspended in 99 vol. (w/v) of 50 nM Tris–HCl buffer and preincubated at 25° for 30 min with [^{3}H]clonidine in a final suspension of 100 vol. The dissociation reaction was initiated by the addition of unlabeled clonidine to a final concentration of 100 nM or 1000 nM (calf and chicken respectively) or by the addition of unlabeled (−)-norepinephrine in a final concentration of 10,000 nM. The reaction was terminated by rapid filtration. Association assays employed the same resuspension of cortical homogenate followed by addition of [^{3}H]clonidine to initiate the reaction. Both association and dissociation assays were run by two similar methods. In the first, the homogenate was contained in a large vessel, and 2.0-ml aliquots were removed at specified times. In the second method, reactions in a series of 2-ml volumes in individual glass tubes were initiated and terminated independently. Rinsing and counting of the filters were identical to that for the equilibrium binding case.

Treatment of data. In all cases experimental data were fitted to mathematical models by the method of nonlinear least squares [14]. For the analysis of saturation binding experiments and the determination of Hill parameters, a weighted nonlinear least squares analysis was employed. The weights were set equal to the inverse of the variances calculated from triplicate determinations. The kinetics and radioligand binding inhibition determinations were analyzed using unweighted data.

For saturation radioligand binding experiments, the model employed was:

$$[B] = [L]\,B_{\max_1}/(K_{d_1} + [L]) + [L]\,B_{\max_2}/(K_{d_2} + [L]) \quad (1)$$

where $[B]$ is the total amount of specifically bound radioligand at a concentration $[L]$, and K_{d_i} and $B_{\max_i}$ are parameters representing the dissociation constant and maximum binding capacity associated with site i. The "extra sum of squares" test [15, 16] was used to decide if inclusion of the second site made a statistically significant improvement in the fit of the model to the data. If it did not, the data were fitted to a one-site model. Scatchard plots were also constructed as an aid to visualizing the binding process but were not used to calculate the final reported binding parameters.

Hill parameters were determined directly from equation 2 by weighted nonlinear least squares.

$$[B] = B_{\max}[L]^{n_H}/(K_d + [L]^{n_H}) \quad (2)$$

Again, standard Hill plots, as shown in Fig. 1, were generated to visualize the data, but were not employed for parameter estimation.

Kinetics of radioligand dissociation and association were fitted by equations 3 and 4 respectively.

$$[B] = C_1 e^{-k_{-1}t} + C_2 e^{-k_{-2}t} + C_\infty \quad (3)$$

$$[B] = C_1(1 - e^{-k_1 t}) + C_2(1 - e^{-k_2 t}) + C_0 \quad (4)$$

For equation 3, $[B]$ is the total bound radioligand at time t, C_1 and C_2 represent the amounts of bound radioligand initially associated with the fast and slow displacement steps, k_{-1} and k_{-2} represent the corresponding apparent first-order rate constants for dissociation, and C_∞ represents the final remaining bound radioligand. The parameters for association (equation 4) are defined in an equivalent manner, except that the rate constants are pseudo first-order constants determined in the presence of a fixed radioligand concentration, and C_0 is the initial concentration of bound radioligand. As above, if a statistical analysis showed that the second term was not significant, it was eliminated and the data were fitted by the corresponding one-exponential model.

Data for inhibition of binding of a fixed concentration of radioligand from cortical membrane suspensions by unlabeled clonidine analogs were fitted to equation 5

$$\%I = 100[C]/(\mathrm{IC}_{50} + [C]) \quad (5)$$

where $\%I$ is the observed inhibition of specifically bound radioligand in the presence of a concentration $[C]$ of unlabeled clonidine analog. Inhibition constants for the clonidine analogs were then calculated from the Cheng–Prusoff relationships [17], equation 6:

$$K_i = \mathrm{IC}_{50}/(1 + [L]/K_d) \quad (6)$$

RESULTS

[^{3}H]Clonidine equilibrium binding. A binding isotherm for the interaction of [^{3}H]clonidine with chicken cerebral membrane suspension (CMS) is shown as both a saturation curve and a Scatchard plot in Fig. 1A. The specific binding was saturable and linear in protein concentration over the range 58–2300 μg protein/ml. The specific binding was eliminated upon denaturation of the protein by boiling (data not shown). Most assays involving chicken CMS utilized tissue which had been frozen previously and maintained at −75°, but freshly prepared tissue

was also investigated and demonstrated binding properties identical to those seen with frozen material. The binding isotherm was the same, within experimental error, when either saturating unlabeled clonidine or norepinephrine was used to determine specific binding.

Figure 1A also presents the binding isotherm in the presence of 10 μM GppNHp. This nonhydrolyzable nucleotide displaced a maximum of 70 ± 2% (N = 3, data not shown) of the bound clonidine. In the presence and absence of the guanyl nucleotide, the binding appeared to be monophasic, involving only a single site. Values of the apparent [^{3}H]clonidine receptor dissociation constant, K_d, and the apparent maximum receptor occupancy, B_{max}, were determined as described in the "Treatment of Data" section and are presented in Table 1. Inclusion of a second K_d and B_{max} term did not lead to a statistically significant improvement in the fit of the data. This conclusion is reinforced by the linearity of the Scatchard plots. GppNHp increased the K_d from 3.4 to 8 nM and decreased B_{max} from 106 to 78 fmoles/mg protein. Lowry protein determinations yielded a value of 58 μg protein/mg tissue, so these two B_{max} values were equivalent to 6.1 and 4.5 pmoles/g tissue respectively. The Hill plots were linear, with Hill parameters increasing from 0.92 ± 0.05 in the absence to 1.0 ± 0.1 in the presence of 10 μM GppNHp.

The corresponding binding isotherm for the interaction of [^{3}H]clonidine with calf CMS (Fig. 1B) revealed a heterogeneous binding pattern in the absence of added GppNHp. Again the binding isotherm was the same when either unlabeled clonidine or norepinephrine was used to determine specific binding. Inclusion of a second pair of parameters representing a lower affinity binding site was always significant at greater than the 99% level (N = 5).

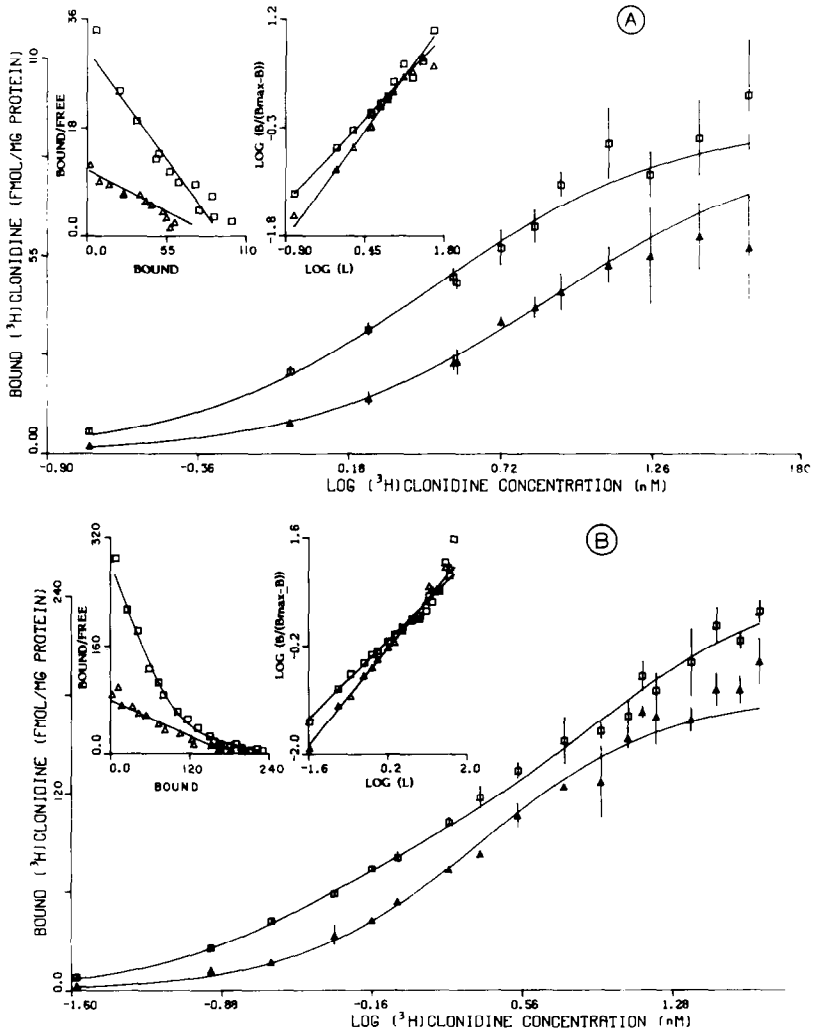


Fig. 1. Specific binding of [^{3}H]clonidine to chicken (A) and calf (B) cerebral homogenates in the absence (□) and presence (▲) of added 10 μM GppNHp. The vertical bars represent the estimated standard deviation of the specific binding based on triplicate replications at each concentration. The two inset graphs for each species are the Scatchard and Hill transformations of the saturation binding data. The data for Fig. 1 are from a single determination conducted twice for both species in the presence of 10 μM GppNHp and four (chicken) or five (calf) times in its absence.

Table 1. Apparent equilibrium parameters for the binding of [^{3}H]clonidine to cerebral cortical homogenates*

Species	[GppNHp] (μM)	K_{d1} (nM)	B_{max1} $\left(\frac{\text{fmoles}}{\text{mg protein}}\right)$	K_{d2} (nM)	B_{max2} $\left(\frac{\text{fmoles}}{\text{mg protein}}\right)$	n_H
Chicken	0	3.4 ± 0.2	106 ± 4			0.92 ± 0.05
	10	8 ± 2	78 ± 10			1.0 ± 0.1
Calf	0	0.38 ± 0.04	92 ± 8	5.9 ± 0.9	210 ± 10	0.82 ± 0.05
	10			3.0 ± 0.2	198 ± 4	0.96 ± 0.04

* Values are averages of two determinations for both species in the presence of added 10 μM GppNHp and four (chicken) or five (calf) determinations in its absence.

This conclusion was reinforced in all cases by the observation of non-linear Scatchard plots. The Hill plot of the data (Fig. 1B inset) was nonlinear, with an average slope significantly less than 1.0. The effect of GppNHp on the specific binding of [^{3}H]clonidine to calf CMS was to displace a maximum of 63 ± 11% (N = 3, data not shown) of the specifically bound radioligand. The saturation curve for [^{3}H]clonidine binding to calf CMS in the presence of 10 μM GppNHp (Fig. 1B) revealed the elimination of the high-affinity binding site by this nucleotide. The K_d and B_{max} parameters computed for the remaining site have values near those of the low affinity site found in the absence of GppNHp. The Hill parameter increased from 0.82 ± 0.03 in the absence of GppNHp to 0.96 ± 0.04 in its presence. All K_d, B_{max}, and n_H parameters computed for the equilibrium binding of [^{3}H]clonidine to chicken and calf CMS are collected in Table 1. The apparent K_d and B_{max} values for calf CMS in the absence of guanyl nucleotides were in reasonable agreement with our earlier results [1] and with the corresponding results at 23° recently reported by Braunwalder, *et al.* [12].

To compare further the ligand specificities of the avian and bovine clonidine binding sites, we determined the abilities of a series of unlabeled clonidine derivatives to inhibit the binding of a fixed concentration of radiolabeled clonidine to cerebral cortical homogenates of the two species. The compounds (Table 2) either have been described previously [11, 18–20] or will be described shortly (R. L. Tolman, manuscript in preparation). Under these conditions, the competition data, in general, fit the model for displacement of the radioligand from a single site. The pseudo-Hill slope parameter was 0.96 ± 0.12 and 0.96 ± 0.09 for chicken and calf respectively. Values of pK_I [$= -\log(K_I)$] for inhibition of [^{3}H]clonidine binding to calf CMS are plotted vs those from chicken in Fig. 2. The correlation coefficient relating the two sets of results is 0.986. Using a linear least squares analysis, one obtains the relation:

$$pK_I(\text{calf}) = 0.644 + 1.03\, pK_I(\text{chicken}), \quad (7)$$

which implies that under these conditions the average clonidine analog has a 4.4-fold greater affinity for the calf α_2 binding site than for the corresponding site in chicken CMS.

Kinetics of the [^{3}H]clonidine receptor interactions. The kinetics of dissociation and association of [^{3}H]clonidine to the avian cortical homogenates (Fig. 3A) were more complex than would be expected from the apparent monophasic equilibrium process seen in Fig. 1A. Both kinetic processes were clearly biphasic in the absence of 10 μM GppNHp. Since the radioligand dissociation process was first-order, and the association was pseudo first-order, both may be represented by models employing sums of exponentials, equations 3 and 4. For dissociation, identical results were obtained when either saturating clonidine or (−)-norepinephrine was employed to displace the radioligand. The addition of 10 μM GppNHp to chicken CMS eliminated the slow step of the dissociation process and significantly decreased, but did not eliminate, the slow association step. The apparent rate constants and relative radioligand occupancies associated with each kinetic step are given in Table 3.

The analysis of the kinetics of [^{3}H]clonidine interaction with the binding sites of calf CMS (Fig. 3B), yielded similar results: two-exponential models were required in the absence of GppNHp, but in this case both the association and dissociation slow steps were

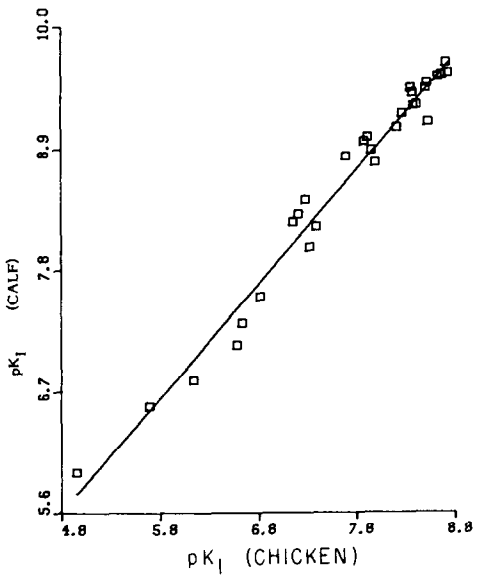
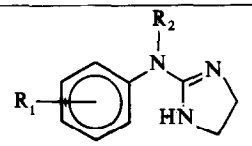


Fig. 2. Comparison of the inhibition of binding of [^{3}H]clonidine to chicken and calf cerebral homogenates by the clonidine analogs of Table 2. The straight line represents the best fit of the data by linear least squares to equation 7.

Table 2. Inhibition of [^{3}H]clonidine binding by clonidine analogs*



	Trivial name	$-\log(K_I)$ (M) Chicken	Calf
R_1 = 2,6-Dichloro, R_2 = H	Clonidine	8.42	9.29
R_1 = 2-Chloro, R_2 = H		7.70	8.82
R_1 = 4-Chloro, R_2 = H		7.28	8.43
R_1 = 2,6-Diethyl, R_2 = H	ST91	8.00	8.77
R_1 = 2,6-Dimethyl, R_2 = H	Xylonidine	8.36	9.44
R_1 = 2-Methyl, R_2 = H	Tolonidine	7.92	9.00
R_1 = 4-Chloro-2-methyl, R_2 = H		8.51	9.44
R_1 = 2,3-Dimethyl, R_2 = H		8.38	9.40
R_1 = 2-Fluoro, R_2 = H		7.22	8.30
R_1 = 2,4-Dimethyl, R_2 = H		8.72	9.68
R_1 = 2-Methoxy, R_2 = H		7.16	8.23
R_1 = 3-Chloro-2-methyl, R_2 = H		8.74	9.58
R_1 = 6-Chloro-2-methyl, R_2 = H		8.64	9.55
R_1 = 2,4,6-Trimethyl, R_2 = H		8.68	9.57
R_1 = 2-Ethyl, R_2 = H		8.22	9.09
R_1 = 5-Fluoro-2-methyl, R_2 = H		7.89	8.96
R_1 = 3-Acetyl-2-methyl, R_2 = H		6.64	7.31
R_1 = 4-Carboxymethyl-2-methyl, R_2 = H		6.58	7.11
R_1 = 5-Acetyl-2-methyl, R_2 = H		6.82	7.55
R_1 = 2-Chloro-4-methyl, R_2 = H		8.52	9.50
R_1 = 2-Chloro-4-methoxymethyl, R_2 = H		7.33	8.00
R_1 = 2,6-Dimethyl-4-hydroxy, R_2 = H		7.96	8.89
R_1 = 2,6-Dimethyl-4-methoxy, R_2 = H		7.40	8.19
R_1 = 2-Chloro-4-diethylaminomethyl, R_2 = H		6.14	6.80
R_1 = 2-Chloro-3-methyl, R_2 = H		8.28	9.22
R_1 = 2,6-Dichloro-4-nitro, R_2 = H		5.70	6.57
R_1 = 4-Amino,2,6-dichloro, R_2 = H		8.54	9.14
R_1 = 3-Amino-2,6-dichloro, R_2 = H		8.39	9.28
R_1,R_2 = Ethylene (2-indoline)		4.96	5.96

* Values of K_I were obtained from IC_{50} values for the inhibition of [^{3}H]clonidine binding as described in the text. Each value of pK_I represents the average of two determinations conducted at five triplicate sets of concentrations.

eliminated in the presence of 10 μM GppNHp. Once again, the dissociation process was the same whether initiated by saturating unlabeled clonidine or (−)-norepinephrine. Our results are in excellent qualitative agreement with the biphasic association and dissociation kinetics of the calf CMS [^{3}H]clonidine interactions recently reported by Braunwalder *et al.* [12].

In all cases where a two-exponential fit is reported, the addition of the second term was significant at the 99% level for each determination. The values of the calculated rate constants, however, showed a substantial variance when separate determinations were compared. This is especially true for the fast association and dissociation steps, as can be seen by the spreads in their values reported in Table 3. The errors of the relative occupancies of the sites associated with the fast and slow steps were smaller.

DISCUSSION

The present investigation, as well as previous reports by us [1] and others [12], reveals that the interaction of [^{3}H]clonidine with calf CMS involves two subsites within the α_2 receptor binding site. While the initial study of the association of this radioligand with chicken CMS revealed an apparently monophasic binding pattern, a more complete investigation and comparison with the calf results suggest that two α_2 subsites of different affinity were also present.

The results of both equilibrium and kinetics determinations of the interaction of [^{3}H]clonidine with cerebral membrane suspensions of the two species were very similar. At equilibrium, the presence of 10 μM GppNHp produced for both species a reduction in the observed binding capacity, an increase in the apparent dissociation constant, and an increase in the calculated Hill parameter. Kinetically, the guanyl nucleotide completely eliminated the slow dissociation step for both species and eliminated the slow association step for calf and sharply reduced it for chicken. In the case of calf CMS, these effects were clearly due to the elimination of the high affinity subsite. The same effect in chicken CMS is consistent with the elimination of a similar high affinity site which was not resolved by a saturation binding experiment. Note that the total binding capacity (Table 1) observed for [^{3}H]clonidine binding to calf CMS, 302 fmoles/mg protein, was three times greater than the corresponding value of 106 fmoles/mg protein observed for chicken CMS. This lower binding

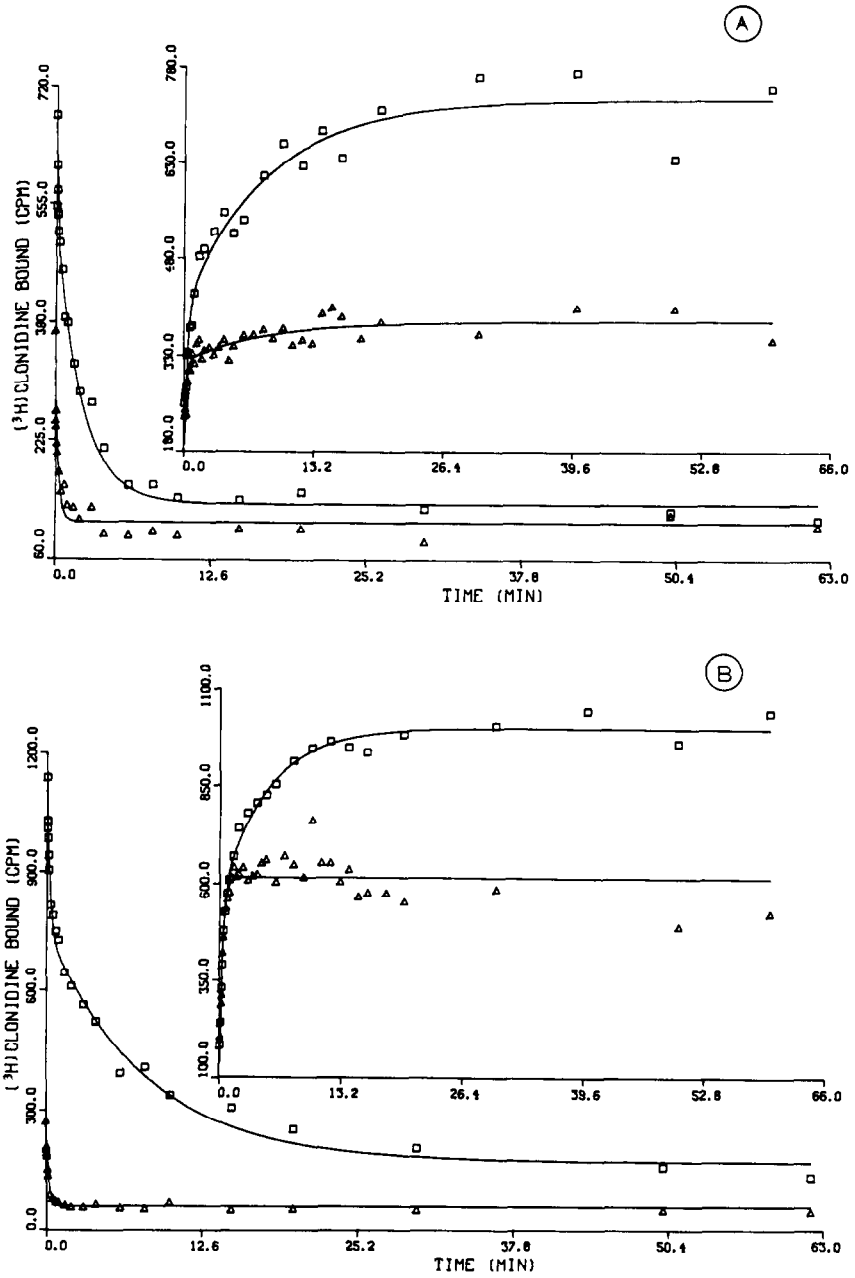


Fig. 3. Kinetics of [^{3}H]clonidine dissociation from, and association to (inset), cerebral homogenates of chicken (A) and calf (B) in the absence (□) and presence (▲) of 10 μM GppNHp. Details of the procedure are given in Materials and Methods. Each set of data represents a single determination which was conducted twice for both species in the presence of 10 μM GppNHp and four times in its absence.

capacity might easily prevent the chicken CMS high affinity site from being resolved by a saturation binding experiment.

The second-order rate constant for association of the radioligand at the ith site, k_i, may be calculated from the first-order rate constant for dissociation, k_{-i}, and the observed pseudo first-order rate constant for association, k_i^{obs}, by means of the expression $k_i = [k_i^{obs} - k_{-1}]/[L]$. Under the conditions of our kinetics determinations, however, the observed association and dissociation constants were nearly equal and subject to considerable experimental error. For this reason, meaningful estimates of the second-order rate constants cannot be made from our results.

It is interesting to note that these results with chicken cerebral CMS closely parallel literature results obtained with rat cerebral CMS. Here, also, saturation binding curves did not always reveal the existence of two binding sites [8, 10], while two sites were determined by analysis of the association and

Table 3. Apparent kinetic parameters for [^{3}H]clonidine binding to cerebral cortical homogenates*

Process	Parameter	Species: Chicken [GppNHp] (μM) 0	Chicken [GppNHp] (μM) 10	Calf [GppNHp] (μM) 0	Calf [GppNHp] (μM) 10
Association	C_1(%)	39 ± 4 (34–43)	68 ± 9 (62–74)	48 ± 7 (40–54)	51 ± 11† (44–60)
	k_1^{obs} (min^{-1})	4 ± 4 (3–35)	7.5 ± 3.5 (6–11)	3.6 ± 3.0 (2.4–10)	4.0 ± 0.8 (3.5–4.6)
	C_2(%)	61 ± 4 (57–66)	32 ± 9 (26–38)	51 ± 7 (46–60)	
	k_2^{obs} (min^{-1})	0.2 ± 0.3 (0.12–0.69)	0.2 ± 0.1 (0.12–0.46)	0.15 ± 0.03 (0.13–0.2)	
Dissociation	C_1(%)	37 ± 8 (26–48)	38 ± 3† (38–39)	40 ± 11 (31–47)	24 ± 13† (22–40)
	k_1^{obs} (min^{-1})	8 ± 11 (4–27)	4.4 ± 0.9 (3.9–4.7)	3.7 ± 2.3 (2.2–7.4)	4.6 ± 2.0 (2.5–5.3)
	C_2(%)	63 ± 8 (52–74)		60 ± 11 (53–69)	
	k_2^{obs} (min^{-1})	0.32 ± 0.13 (0.17–0.49)		0.17 ± 0.03 (0.12–0.20)	

* The apparent kinetic parameters were obtained from the best fit of the data to equation 3 for dissociation or equation 4 for association. The values of each parameter are the average of two determinations in the presence of 10 μM GppNHp and four determinations in its absence. The figures in parentheses represent the ranges of the parameters. The indicated error estimate is based on half of the range of the data (N = 2) or the standard deviation of the data (N > 2).

† The percent occupancy of the radioligand in the presence of 10 μM GppNHp was calculated relative to the observed total binding in the absence of this nucleotide.

dissociation kinetics. The elimination of the high affinity site by a guanyl nucleotide, GTP, has been reported for rat CMS [10].

The excellent correlation observed for the inhibition of [^{3}H]clonidine binding to chicken and calf CMS by a series of clonidine analogs once again reinforces the similarity of the α_2 receptors for the two species. At the radioligand concentrations employed (0.2 nM for calf and 1.0 nM for chicken), the binding would be predominantly to the high affinity subsite (82% for calf and 76% for chicken).

The apparent dissociation constant observed for avian cerebral homogenates in the absence of added guanyl nucleotide represents contributions from both the low affinity subsite seen in the presence of 10 μM GppNHp and the unresolved high affinity subsite. If the contribution of the low affinity subsite is subtracted, it can be shown that approximately 80% of the high affinity sites must be bound at the apparent K_d of 3.4 nM. Therefore, the actual K_{d_1} of the high affinity site must be <1 nM. These derived values, together with those of calf and values previously reported for the rat cerebral CMS by three groups of investigators [8–10], are shown in Table 4. It is clear from this table that the properties of the central α_2 receptors in the avian species resemble those of the two mammalian species.

The effect of GppNHp on calf and chicken CMS

Table 4. Two-site binding parameters for three species*

Species	High affinity site K_{d_1} (nM)	High affinity site B_{max_1} (fmoles/mg protein)	Low affinity site K_{d_2} (nM)	Low affinity site B_{max_2} (fmoles/mg protein)
Chicken	<1	28	8	78
Calf	0.38	92	5.9	210
Calf†	1.1	82	5.4	181
Rat‡	0.5	26§	3	168
Rat‖	1.5	83	4	180
Rat¶	0.4	5.2	6.1	68

* The values for chicken and calf are based on the data and calculations presented in this paper. The values for rat are those presented by others who did detect two [^{3}H]clonidine binding sites in rate brain.

† Values of Braunwalder *et al.* [12].

‡ Values of U'Prichard *et al.* [8].

§ The authors' B_{max} values were converted from pmoles/mg tissue to fmoles/mg protein assuming the ratio 58 μg protein/mg tissue found in the present investigation.

‖ Values of Rouot *et al.* [10].

¶ Values of Hammer *et al.* [9].

appears to be an elimination of the high affinity subsite, with no effect on the K_d or B_{max} of the remaining low affinity subsite. A similar response to GTP has been seen by others for rat [10] and calf [12] brain homogenates. These results are consistent with a model in which a distinct high affinity subsite reversibly binds the guanyl nucleotide to form a complex with negligible affinity for clonidine, such as the α_0 subsite postulated by Glossmann and Hornung [21] for rat brain α_2 adrenoceptors. The data are not consistent with a model in which the high affinity subsite is rapidly and reversibly converted to the observed low affinity subsite. Such a model has been suggested for peripheral α_2 adrenoceptors [22]. If this latter model held, GppNHp would produce no net change in the total apparent B_{max}, since the reduction in B_{max_1} would be accompanied by a concomitant increase in B_{max_2}.

The results of this study thus emphasize the similarity of the interaction of [^{3}H]clonidine with specific binding sites in the avian and mammalian species. The observation of a monophasic saturation binding curve for a radioligand is shown to be insufficient to rule out the existence of receptor subtypes, since these may be detected upon the application of guanyl nucleotides or by kinetic techniques.

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