RESEARCH ARTICLES

NO EVIDENCE FOR THE ACTIONS OF 'CLONIDINE-DISPLACING SUBSTANCE' ON ALPHA2-ADRENOCEPTORS

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ABSTRACT

It has been suggested that 'clonidine-displacing substance' (CDS) is a putative endogenous substance at imidazoline receptor. CDS was found to recognize not only the imidazoline binding sites but also α_2-adrenoceptors. However, the information of the functional activities of CDS at the α_2-adrenoceptor is still limited. The aim of this study was to examine the properties and functions of CDS, in particular, the actions at α_2-adrenoceptors. In the present study, the methanolic CDS extracts were prepared from bovine brain. CDS activity was determined by the amount of the extract that displaced 50% of $[^3H]$-clonidine binding to bovine cerebral cortex membranes. CDS activity from bovine brain methanolic extract was 4.8 ± 0.5 units/g wet weight. Based on radioligand binding assays, this extract recognized both α_2-adrenoceptors on bovine cerebral cortex membranes labeled by $[^3H]$-clonidine and non-adrenoceptor imidazoline binding sites on porcine renal cortex membranes labeled by $[^3H]$-idazoxan. The CDS extract was found to be slightly more potent (2 folds) at α_2-adrenoceptors than at non-adrenoceptor imidazoline binding sites. Functional studies of CDS were examined in the models of α_2-adrenoceptor-mediated inhibition of forskolin-stimulated cAMP accumulation in guinea-pig cerebral cortex slices and in porcine isolated palmar lateral vein. The effects were compared to those of the known α_2-adrenoceptor agonists, UK14304 and antagonized by the α_2-adrenoceptor antagonist, idazoxan. The results from this study showed that bovine brain methanolic CDS extract did not display any activity involving α_2-adrenoceptors as predicted from radioligand binding. In conclusion, CDS is able to displace clonidine from α_2-adrenoceptors but no related biological activity was detected at this sites. Whether the possibility that CDS is an endogenous ligand at α_2-adrenoceptors and at non-adrenoceptor imidazoline binding sites and possesses any significant activities at these sites remain to be clarified.

Key words : clonidine-displacing substance (CDS), alpha2-adrenoceptor, imidazoline receptor

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Clonidine-displacing substance ไม่มีผลต่อ alpha2- adrenoceptor

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บทคัดย่อ

Clonidine-displacing substance (CDS) เป็นสารที่สังเคราะห์ขึ้นในร่างกายสามารถจับกับตัวรับ imidazoline และ α_2-adrenoceptor เนื่องจากข้อมูลการศึกษาเกี่ยวกับผลของ CDS ต่อตัวรับ α_2 -adrenoceptor ยังมีจำกัด งานวิจัยครั้งนี้จึงมีวัตถุประสงค์เพื่อตรวจสอบคุณสมบัติและหน้าที่ของ CDS ต่อตัวรับ α_2-adrenoceptor ในการศึกษาครั้งนี้ใช้สารสกัด CDS จากสมองวัวด้วย methanol ฤทธิ์ของ CDS วัดโดยปริมาณของสารสกัดที่แทนที่การจับของ [^{3}H]-clonidine กับเนื้อเยื่อของสมองวัวส่วน cortex ได้ 50% มีค่าเท่ากับ 4.8±0.5 หน่วยต่อกรัม ผลจากการศึกษาการจับของสารสกัดกับตัวรับ α_2-adrenoceptor ของเนื้อเยื่อสมองวัวส่วน cortex ที่ถูกจับด้วย [^{3}H]-clonidine และการจับของสารสกัดกับตัวรับ non-adrenoceptor imidazoline ของเนื้อเยื่อ renal cortex ของหมูที่ถูกจับด้วย [^{3}H]-idazoxan ด้วยวิธี radioligand binding assay พบว่าสารสกัด CDS สามารถจับกับตัวรับ α_2-adrenoceptor ได้มากกว่าตัวรับ non-adrenoceptor imidazoline 2 เท่า ศึกษาการทำงานของสารสกัด CDS โดยรูปแบบของการยับยั้งการสะสมของ cAMP ที่ถูกกระตุ้นด้วย forskolin ใน cerebral cortex slices ของหนูตะเภาและ isolated palmar lateral vein ของหมูที่มีกลไกผ่าน α_2-adrenoceptor เปรียบเทียบผลที่เกิดขึ้นกับ UK14304 ซึ่งเป็น agonist ของตัวรับ α_2-adrenoceptor และ idazoxan ซึ่งเป็น antagonist ของตัวรับ α_2-adrenoceptor จากผลการทดลองแสดงให้เห็นว่าสารสกัด CDS จากสมองวัวด้วย methanol ไม่มีฤทธิ์ผ่าน α_2-adrenoceptor โดยสรุปแล้วพบว่า CDS สามารถแทนที่ clonidine จากตัวรับ α_2-adrenoceptor แต่ไม่แสดงฤทธิ์ทางชีววิทยาใด ๆ การศึกษาต่อไปจำเป็นต้องตรวจสอบความเป็นไปได้ของการเป็น endogenous ligand ของ CDS ที่ตัวรับ α_2-adrenoceptor และ non-adrenoceptor imidazoline ตลอดจนฤทธิ์ของ CDS ต่อตัวรับดังกล่าว

คำสำคัญ : clonidine-displacing substance (CDS), alpha2-adrenoceptor, imidazoline
             receptor

INTRODUCTION

"Clonidine-displacing substance" (CDS) was first isolated from rat and calf brains by Atlas and Burstein[1,2] and it was suggested to be a candidate for an endogenous ligand at non-adrenoceptor imidazoline binding site (imidazoline receptor). A partially-purified extract of the brain was found to displace specifically bound [3H]-clonidine from binding sites on rat brain or bovine cerebral cortex membranes, which were catecholamine-sensitive, α_2-adrenoceptor binding sites, and hence the active agent was called clonidine-displacing substance[1]. CDS has been shown to interact with non-adrenoceptor imidazoline binding sites in several tissues such as brain which are defined by [3H]-*para*-aminoclonidine[3], and in rabbit kidney labelled by [3H]-idazoxan[4]. CDS has been detected in several tissues such as brain[5], kidney[6], adrenal chromaffin cells[7,8], liver[9] and lung[10]. However, the chemical structure of the active substance remains to be elucidated.

Although CDS has been shown to have an affinity for both α_2-adrenoceptors and imidazoline binding sites, its biological activities remain unclear. At α_2-adrenoceptors, CDS extract was reported to inhibit electrically-stimulated twitch responses in rat vas deferens and the inhibitory effect was partially reversed by the α_2-adrenoceptor antagonist, yohimbine[11]. Another evidence of CDS action at α_2-adrenoceptors was inhibitory adrenaline-induced aggregation but potentiating ADP-induced aggregation of human platelets, like clonidine[12]. However, there has been no other evidence support the action of CDS at α_2-adrenoceptors since then.

For CDS action at imidazoline binding sites, CDS was found to contract rat gastric fundus strips, the site that was non-catecholamine, since this effect of CDS was not blocked by α_2-adrenoceptor antagonists[13]. Brain-derived CDS extract has been reported to modulate arterial blood pressure through the non-catecholamine site when injected directly into the ventrolateral medulla[14,15]. However, the results examined by these 2 groups showed opposite effects, the former lowered blood pressure whereas the latter caused elevated blood pressure. Another CDS action was the induction of substantial release of catecholamines from cultured chromaffin cells[7-8]. Whether CDS can be a putative endogenous substance that acts at both sites, the exact biological action of CDS remains to be investigated.

The aims of this study are to examine whether the bovine brain extracts possess any agonist activities at functional α_2-adrenoceptors, corresponding to their binding properties. In this study, the binding characteristics of crude methanolic CDS extracts and functional activities at α_2-adrenoceptors were examined. Two α_2-adrenoceptors models were used, i.e. central α_2-adrenoceptors on guinea-pig cortex slices which mediate inhibition of forskolin-stimulated cyclic AMP[16] and α_2-adrenoceptors on porcine palmar lateral veins, the activation of which results in inhibition of forskolin-stimulated cyclic AMP[17].

MATERIALS AND METHODS

Chemicals, drugs and solutions

(-)-Noradrenaline bitartrate, forskolin, Dowex-50, alumina, Tris-HCl, EDTA, idazoxan (2-(2(1,4-benzodioxanyl))2-imidazoline hydrochloride) and salts in Krebs-Henseleit buffer (NaCl, KCl, CaCl$_2$, NaHCO$_3$, MgSO$_4$, KH$_2$PO$_4$ and glucose) were purchased from Sigma Chemical Company. Other chemical used included cirazoline (S.M. Chemical Company), UK14304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline bitartrate, Pfizer), rolipram (Schering) and HPLC grade methanol (Fisons). [3H]-clonidine hydrochloride (specific activity of 30 Ci/mmol), [3H]-idazoxan ([3H]-RX781094, specific activity 43 Ci/mmol), [3H]-adenine (specific activity 851 GBq/mmol) and [14C]-cAMP (specific activity 1.6 GBq/mmol) were from Amersham. Scintillation cocktails (Emulsifier Scintillator 299, Packard) were also included.

All drugs were dissolved in distilled water and added in volumes less than 1% of the K-H solution for cAMP assays except forskolin and rolipram which were dissolved in absolute alcohol in volumes less than 3% of assay volume and then diluted with Krebs'solution.

Preparation of clonidine-displacing substance (CDS) from bovine brain

Bovine brain was obtained from a local abattoir immediately after slaugther of the animal. The brain was kept on ice during transportation. Half a bovine brain (110-160 g wet weight) was taken and the cerebellum and all pia mater removed prior to chopping into small pieces. Tissues were homogenized in an

OMNI-GEN sealed homogenizer (setting 4.5 for 3x3 min) with 5 volumes (w/v) of distilled water. The resulting homogenate was centrifuged at 65,000g for 30 min at 4°C (MSE Superspeed 65). The supernatant was then removed, boiled for approximately 40-60 min to precipitate soluble protein and to reduce the volume and then allowed to cool to room temperature. The resulting solution was recentrifuged at 65,000g at 4°C for 20 min. The supernatant was removed from pellets, frozen at -20°C and then freeze-dried. The freeze-dried material was used for further extraction. The lyophilysed material was extracted by sonication with 2x20 volume (w/v) of highly purified methanol (HPLC grade) at room temperature for 30 min. The methanolic extracts were combined and centrifuged at 3,000 rpm for 5 min (MSE Mistral 3000) to remove any particulate matter and evaporated to dryness in a rotary evaporator at low pressure. The residual material was dissolved in 10 volumes (w/v) of doubled distilled water, frozen and lyophilysed again to remove all trace of organic solvent. The final lyophilysates were redissolved in 10 volumes (w/v) doubled distilled water (3 ml) and kept at -20°C until required for use.

Membrane preparation

Bovine cerebral cortex membrane preparation[18]

Calf brains were obtained from local abattoir immediately after slaughter of the animals. The cerebral cortices were homogenized in 20 volumes of ice-cold Tris buffer (50 mM Tris HCl; pH 7.7 at 25° C) using an OMNI-GEN sealed macro-homogenizer (Model N017506, Camlab Ltd.) (setting 5;120 sec) to minimize potential risk associated with aerosol formation. The homogenate was then centrifuged at 20,000 rpm (40,000g) for 10 min at 4° C (MSE Europa 24M). The supernatant was discarded and the pellet was resuspended in 20 volumes (w/v) of ice-cold Tris buffer and recentrifuged for a further 15 min and the supernatant discarded. The remaining pellets were weighed and resuspended in 6 volumes of ice-cold 50 mM Tris buffer, pH 7.4, and then aliquoted for either direct use in binding assay or stored at -20° C.

Porcine kidney cortex membrane preparation

This membrane preparation for the non-adrenoceptor imidazoline binding sites was based on the method originally described by Vigne et al.[19] The porcine kidney was obtained from local abattoir soon after slaughter of the animal and kept on ice prior to the dissection of the cortex. Working on ice, the kidney was cut in half and the outer membranes surrounding the kidney was stripped off and the cortex separated from the medulla. The cortex was cut into small pieces and homogenized in 24 volumes of ice-cold Tris buffer (50 mM Tris HCL, 5.0 mM EDTA; pH 7.4) using a Polytron PT (setting 6; 3x30 sec). The homogenate was then poured through a 25 μM nylon mesh to remove large particulate matter. The homogenate was centrifuged (MSE Europa 24 M) at 20,000 g for 15 min at 4°C. The supernatant was discarded and the pellet resuspended in ice-cold Tris buffer (50 mM Tris HCL, 0.5 mM EDTA; pH 7.4) and recentrifuged for further 3 min. The final pellets were resuspended in 10 volumes of Tris assay buffer (50 mM Tris HCl; pH 7.4) and stored at -20°C.

Activity of bovine brain CDS extracts

The CDS activity of the methanolic extracts from bovine brain was evaluated by the ability of the extracts to displace 1 nM [³H]-clonidine binding from α_2-adrenoceptor sites on bovine cerebral cortex membranes. The assay volume of 0.5 ml, containing 100 μl bovine cerebral cortex membrane (200-300 μg protein) in 50 mM Tris-HCl buffer (pH 7.4), was incubated with 1 nM [³H]-clonidine in the presence of increasing concentrations of CDS extract. Non-specific binding was determined in the presence of 10^{-4}M noradrenaline. After an incubation period of 60 min at 25°C, bound radioactivity was separated from free by vacuum filtration over Whatman GF/B glass fiber filters using a Brandel cell harvester, followed by 2x3 ml washes with ice-cold assay buffer. The filters were then suspended in 4 ml of scintillation cocktail and bound ligands were determined by scintillation spectrometry.

One unit of CDS activity is defined as the amount of the extract that produced 50% inhibition of [³H]-clonidine (1 nM) binding to bovine cerebral cortex membranes in a total volume of 1 ml assay (assay volume was always 0.5 ml). CDS activity was calculated in unit/g wet weight of tissues. Number of units/g tissue wet weight was calculated from

the final redissolved volume with double distilled water, the number of lyophilysed portions were equally divided and the original amount of tissue was used for the extraction.

CDS extract binding to non adrenoceptor binding sites on porcine kidney cortex membranes

Porcine kidney cortex membranes (50 μl, approximately 200 μg protein) were incubated with 1.0 nM [^{3}H]-idazoxan in the presence and absence of increasing concentrations of CDS extract, in a final volume of 0.5 ml buffer (50 mM Tris-HCl; pH 7.4 at 25°C). Non-specific binding was determined in the presence of 10^{-4} M cirazoline (<15% of total binding). After 1 hr of incubation, bound radioactivity was separated from free by vacuum filtration over GF/B filters, using a Brandel cell harvester. The filters were washed with 3x3 ml of ice-cold buffer and suspended in scintillation cocktail, and the radioactivity was determined by liquid scintillation counting.

Functional activities of CDS extract at alpha2-adrenoceptors

The effect of clonidine-displacing substance on forskolin-stimulated cyclic AMP accumulation in guinea-pig cerebral cortex slices

Male guinea-pigs (250-300 g) were killed by cervical dislocation. The cerebral cortices were dissected out on ice and chopped into miniprisms (350x350 μm). The slices were then dispersed into oxygenated Krebs-Henseleit buffer (K-H buffer) in a stoppered conical flask with several cycles of aspiration and re-addition of medium (1 guinea-pig cortex per 100 ml flask containing 30-40 ml medium). The cortex slices were pre-incubated for 60 min in modified Krebs-Henseleit buffer in a shaking water bath at 37° C. After 1 hr equilibation, the slices were washed with K-H buffer and then were labeled with 0.4 μM [^{3}H]-adenine (37 kBq/ml) for 45 min. After washing away the unincorporated [^{3}H]-adenine with 3 changes of K-H saline, the slices were allowed to settle. Aliquots of cortex slices (25μl) were distributed into flat-bottom vials containing K-H saline to maintain a final volume of 300 μl. Basal level was the [^{3}H]-cAMP accumulation when incubated the slices in the K-H saline for 10 min. The forskolin-stimulated effect was the [^{3}H]-cAMP

accumulation when incubated the slices in K-H saline for 10 min and then incubated with 10 μ M forskolin for 10 min before termination with HCl. The effects of the methanolic extracts were compared with that of α_2-adrenoceptor agonist, UK14304. The extract or UK14304 was added and incubated for 10 min before adding 30 μM forskolin for 10 min. Incubation tubes were resealed under an O_2 : CO_2 (95% : 5%) atmosphere after each addition. In the experiment that examined the effect of α_2-adrenoceptor antagonist, idazoxan (10^{-6} M) was added 10 min before the agonist, UK14304, or CDS extracts and incubated for 10 min before the addition of forskolin (30 μ M). Incubations were terminated after 10 min by the addition of 200 μl 1M HCl containing 25-30 Bq [^{14}C]-cAMP. Cold water (750 μl) was added and then the slice suspension was centrifuged at 2000 g for 5-10 min.

[^{3}H]-cAMP was isolated by sequential Dowex50/alumina chromatography using [^{14}C]-cAMP as a recovery marker. In some experiments the effects of brain methanolic CDS extract alone (10 min incubatiou) were investigated. Aliquots of the supernatant (0.9 ml) were applied to Dowex50 columns (Bio-Rad, filled with 1 ml resin). Distilled water (2 ml) was added to elute [^{3}H] ATP and [^{3}H]-ADP to waste. [^{3}H]-cAMP was desorbed from the Dowex resin by applying 4 ml water which was allowed to drip directly onto the alumina columns. [^{3}H]-cAMP was eluted from the alumina columns with 5 ml 0.1 M imidazole directly into scintillation vials. Scintillation cocktail (10 ml) was added to the effluent and mixed to form a gel prior to counting in a refrigerated liquid sciutillation counter, using a dual channel [^{3}H]/[^{14}C] program. [^{3}H]-cAMP levels were corrected for recovery from the Dowex/alumina chromatography and the amount of total [^{3}H]-adenine taken up into each individual tissue.

The effect of clonidine-displacing substance on α_2-adrenoceptor mediated inhibition of forskolin-stimulated cyclic-AMP accumulation in porcine isolated palmar lateral vein

The presence of α_2-adrenoceptor-mediated inhibition of [^{3}H]-cAMP accumulation in the porcine isolated palmar lateral vein has previously been demonstrated by Wright et al, (1995)[17]. Porcine fore-trotters were obtained from a local abattoir and transported on ice to the laboratory. The palmar lateral vein was dissected out, placed in oxygenated, ice-cold K-H saline containing

2% Ficoll, and stored overnight at 4°C. Segments of palmar lateral vein were cut into 5 mm lengths and incubated in modified Krebs-Henseleit saline for 60 min at 37 °C in a shaking water bath. After this period of time segments of vessel were incubated with 0.8 μM [^{3}H]-adenine (75 KBq/ml) again for a period of 60 min at 37°C in a shaking water bath. After incubation the segments of vessel were washed 3 times by resuspension and then one segment was transferred into each flat-bottom plastic incubation vial containing Krebs solution to a final assay volume of 300 μl. Segments were allowed to equilibrate for 20 min and all experiments were carried out in quadruplicate. The cyclic-AMP selective phosphodiesterase inhibitor, rolipram(10^{-5}M) was added to all assay tubes to prevent degradation of cAMP[17]. The sequence of addition of antagonist, agonist or the extracts and forskolin were the same as in the experiment with guinea-pig cortex slice as mentioned above. The α_2-antagonist, idazoxan (10^{-6} M), was added 10 min before the agonist, UK14304, or CDS extracts and incubated for 10 min. Finally, forskolin (30 μM) was added for 10 min. Incubation tubes were resealed under an O_2 : CO_2 (95% :5%) atmosphere after each addition. Incubation was stopped by the addition of 200 μl 1M HCl, followed by 750 μl distilled water.

[^{3}H]-cAMP was separated from [^{3}H]-adenine and other [^{3}H]-products using sequential Dowex/alumina chromatography in Dowex 50 columns (Bio-Rad), adjusting for variation in recovery by the addition of [^{14}C]-cAMP (approximately 30 Bq per tube) as mentioned above. [^{3}H]-cAMP and total tritium in the tissue was determined using scintillation spectrometry.

Data analysis

The inhibition of radioligand binding by CDS extracts was analysed using a non-linear least square method using Kaleidagraph (Synergy software) on a Mac-Intosh computer. In functional assays, the level of forskolin-stimulated [^{3}H]-cAMP accumulation produced by the effect of α_2-adrenoceptor agonist or antagonist or the extracts were substracted with the basal level of cAMP production. The

results were presented as percentages of the control forskolin-stimulation above basal level.

The data shown were mean±standard error of mean (SEM) from at least 4 experiments. Statistical analysis used was the Student's t-test. The difference between mean values was considered statistically significant if p<0.05.

RESULTS

Binding properties of bovine brain methanolic CDS extracts

Following aqueous extraction, deproteinization, freeze drying and further extraction with methanol, 110-160 g wet weight of bovine brain produced a slightly yellow material which was dissolved in 12 volumes of double-distilled water to yield the 'methanolic CDS extract'. As shown in figure 1, the brain CDS extract could recognize both α_2-adrenoceptors and non-adrenoceptor imidazoline binding sites. The line with open circle showed that the extract caused a concentration-dependent displacement of 1 nM [^{3}H]-clonidine binding to bovine cerebral cortex membranes which contained α_2-adrenoceptor sites[20]. At the highest concentration examined, the brain extracts (100 μl/ml) caused 80.5±2.5 % inhibition (n=8) of [^{3}H]-clonidine binding. The amount of CDS extract producing 50% inhibition of 1 nM [^{3}H]-clonidine binding (which was equivalent to 1 unit/ml) was 18.6±1.7 μl/ml (n=8). The slope of the displacement curves was 1.09±0.08 (n=8). Based on the wet weight of the original starting material, the activity of CDS from bovine brain was 4.8± 0.5 units/g wet weight (n=8 batches).

The line with closed circle showed that the extract produced a concentration-dependent displacement of [^{3}H]-idazoxan from porcine renal cortex membranes which contained the non-adrenoceptor imidazoline binding sites[19]. The amount of CDS extract producing 50% inhibition of 1.0 nM [^{3}H]-idazoxan was 33.2±3.4 μl/ml (n=4). This result showed that the brain CDS extract was approximately 2 folds more potent at α_2-adrenoceptors than at non-adrenoceptor imidazoline binding sites.
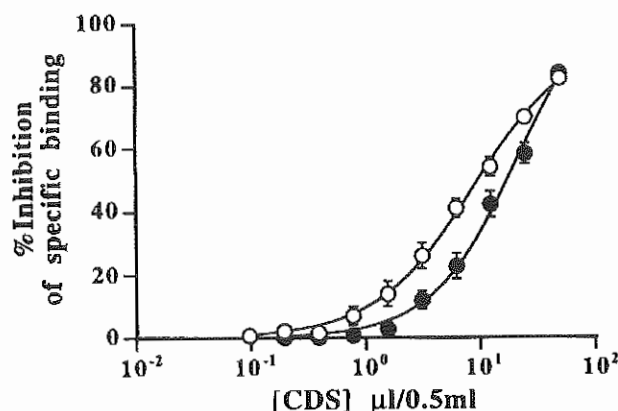
Figure 1 The effect of bovine brain methanolic CDS extracts against 1nM [^{3}H]-clonidine binding on bovine cerebral cortex membranes (O) and against 1nM [^{3}H]-idazoxan binding on porcine renal cortex membranes (●). Non-specific binding was determined in the presence of 10^{-4} M noradrenaline (10-15% of total binding) for the sites labeled by [^{3}H]-clonidine and in the presence of 10^{-4} M cirazoline (<15% of the total binding) for the sites labeled by [^{3}H]-idazoxan. The concentration of the extract is expressed in μl/0.5 ml assay volume. The figures shown are means ± SEM from 4-8 experiments. Mean IC_{50} (n=8) of brain extract against the site labeled by [^{3}H]-clonidine (n=8) is significantly difference at p<0.05 from that of the site labeled by [^{3}H]-idazoxan (n=4) (see also the text).

*Functional activities of CDS extracts at α_2-adrenoceptors*

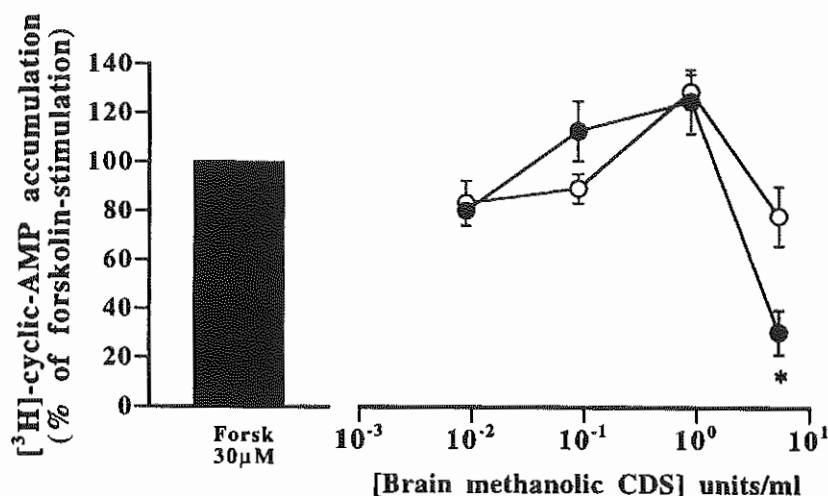*The effects of α_2-adrenoceptor agonist, UK14304, on forskolin-stimulated cyclic AMP accumulation*

The basal level of [^{3}H]-cAMP accumulation in guinea-pig cerebral cortex slices was 1,323 ±196 dpm (n=4). Forskolin (30 μM) caused a 3-4 folds increase in [^{3}H]-cAMP accumulation, while UK14304 (10^{-7} M) did not affect the basal levels (1,425±210 dpm, n=4). However, UK14304 caused a concentration-dependent inhibition of forskolin-stimulated cAMP accumulation, with a pD_2 value of 7.49±0.21 (n=4) (Figure 2). UK14304 caused complete inhibition of forskolin-stimulated [^{3}H]-cAMP accumulation to basal levels. The α_2-adrenoceptor antagonist, idazoxan (10^{-6} M), had no significant effects on either basal or forskolin-stimulated [^{3}H]-cAMP level (Figure 2, left panel) but abolished the inhibitory effect of UK14304 on forskolin-stimulated [^{3}H]-cAMP accumulation in guinea-pig cortex slices (Figure 2, right panel). These experiments showed the presence of α_2-adrenoceptors on guinea-pig cerebral cortex membranes, which were negatively linked to cAMP formation.

The effects of brain methanolic CDS extracts on forskolin-stimulated cyclic AMP accumulation in guinea-pig cortex slices.

Brain methanolic CDS extract (0.9 unit/ml) had no significant effect on basal [^{3}H]-cAMP accumulation (1,485±220 dpm for basal and 1,375±385 dpm for the extract). All the concentrations of brain methanolic extracts examined (0.009-5.3 units/ml) produced no significant change (at p<0.05, n=4) in forskolin-stimulated [^{3}H]-cAMP accumulation. (Figure 3, right panel). The figure appeared to show a biphasic effect but these changes were not significantly different from the control forskolin response. Idazoxan (10^{-6} M) had no significant effects on either basal (1,442±320 dpm) or forskolin-stimulated [^{3}H]-cAMP levels (99±4% of control response). In the presence of 10^{-6} M idazoxan, brain methanolic CDS extract had no significant effect on forskolin-stimulated [^{3}H]-cAMP accumulation. Surprisingly, at a higher concentration (5.3 units/ml), in the presence of idazoxan, the extract significantly inhibited forskolin-stimulated [^{3}H]-cAMP accumulation to 30% of forskolin-stimulation.
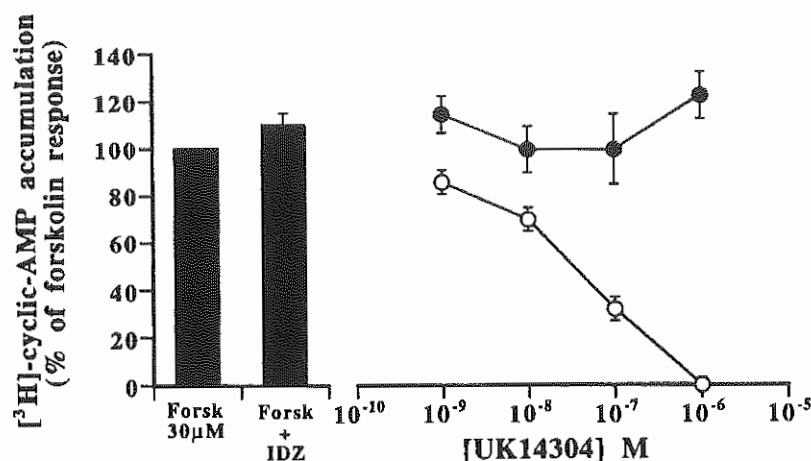
Figure 2. The effect of UK14304 on forskolin-stimulated $[^3H]$-cAMP accumulation in guinea-pig cerebral cortex slices. The left panel (bar) shows the effect of 30 μM forskolin (Forsk) and the effect of 10^{-6} M idazoxan (IDZ) on forskoin-stimulated $[^3H]$-cAMP accumulation above basal level. The right panel represents the effect of UK14304 (O) and UK14304 in the presence of 10^{-6} M idazoxan (●) on 30 μM forskolin-stimulated $[^3H]$-cAMP accumulation in guinea-pig cerebral cortex slices. The level of $[^3H]$-cAMP is shown as percentage of the 30 μM forskolin response (above basal level). Each experiment was carried out in quadruplicate. The values shown are means ± SEM from 4 experiments.

Figure 3. The effect of brain methanolic CDS extracts on forskolin-stimulated $[^3H]$-cAMP accumulation in guinea-pig cerebral cortex slices. The left panel (bar) shows the effect of 30 μM forskolin-stimulated $[^3H]$-cAMP accumulation above basal level which represents 100%. The right panel represents the effects of extract (O) and the extract in the presence of 10^{-6} M idazoxan (●) on 30 μM forskolin-stimulated $[^3H]$-cAMP accumulation in guinea-pig cerebral cortex slices. The level of $[^3H]$-cAMP is shown as percentage of the 30 μM forskolin response. Each experiment was carried out in quadruplicate. The values shown are means ± SEM from 4 observations.
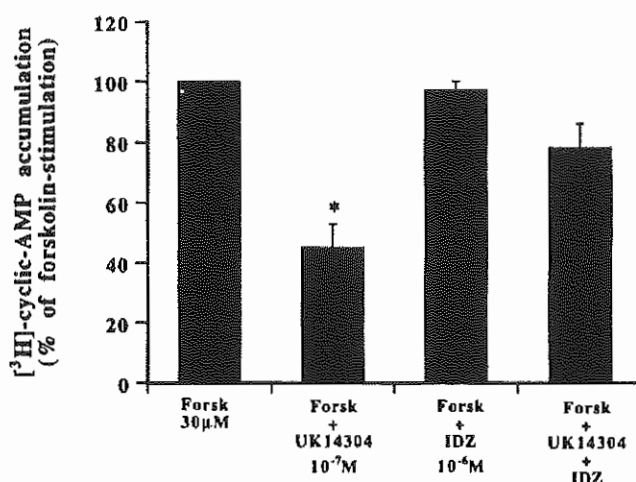
* represents statistical difference from the effect of forskolin- stimulation at $p < 0.05$.

The effects of UK14304 and brain methanolic CDS extracts on forskolin-stimulated cyclic AMP accumulation in porcine palmar lateral veins.

UK14304 (10^{-7} M) had no effect on the basal [^{3}H]-cAMP level but inhibited forskolin-stimulated [^{3}H]-cAMP accumulation in porcine palmar lateral veins to 45±8 % (n=4) of the forskolin response (Figure 4). The inhibitory effect was reduced by 10^{-6} M idazoxan to 78±9 % (n=4). of the forskolin response.

Brain methanolic CDS extract (0.9 unit/ml) had no effect on the basal [^{3}H]-cAMP accumulation (1,008±336 dpm for basal and 840±168 dpm for the response of the extract, n=4). Forskolin (30 µM) produced an (19,280±2,400 dpm) approximate 20 folds increase in [^{3}H]-cAMP accumulation. Figure 5 shows that the extract, over the concentration range of 0.009-5.3 units/ml, does not produce any effect on forskolin-stimulated [^{3}H]-cAMP accumulation, either in the presence or absence of idazoxan (10^{-6} M).

Figure 4. The effect of UK14304 (10^{-7} M) in the presence and absence of 10^{-6} M idazoxan (IDZ) on 30 µM forskolin (Forsk)-stimulated [^{3}H]-cAMP accumulation in porcine palmar lateral vein. The level of [^{3}H]-cAMP is shown as percentage of the 30 µM forskolin response. Each experiment was carried out in quadruplicate. The values shown are means±SEM from 4 experiments.
* represents statistical difference from the effect of forskolin-stimulation at p<0.05.
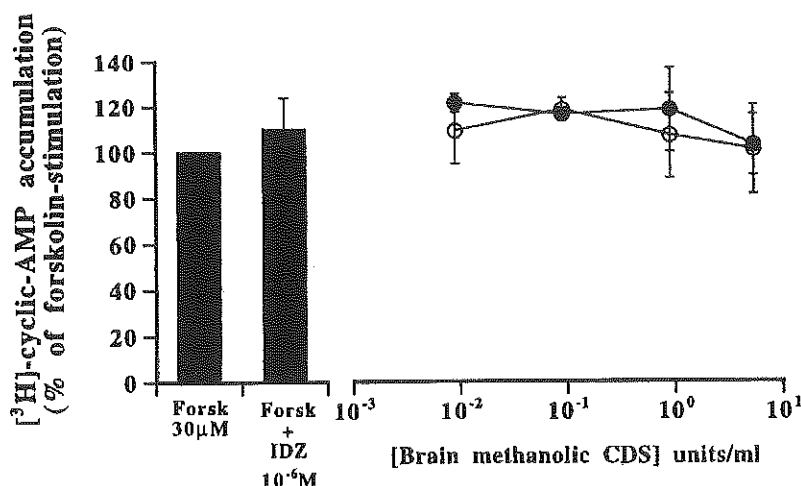
Figure 5. The effect of brain methanolic CDS extract on forskolin-stimulated [^{3}H]-cAMP accumulation in porcine palmar lateral vein. The left panel (bar) shows the effect of 30 μ M forskolin-stimulated [^{3}H]-cAMP accumulation above basal level which represents 100% and the effect of 10^{-6} M idazoxan on forskolin response. The right panel represents the effects of the extract in the absence (O) and in the presence of 10^{-6} M idazoxan (●) on 30 μM forskolin-stimulated [^{3}H]-cAMP accumulation in porcine palmar lateral vein. The level of [^{3}H]-cAMP is shown as percentage of the 30 μM forskolin response. Each experiment was carried out in quadruplicate. The values shown are means±SEM from 4 experiments. There is no significant difference (at p<0.05) between the effects of the extract on forskolin-stimulation and the effect of forskolin-stimulation per se.

DISCUSSION

The present study has confirmed that methanolic extract of bovine brain contains an agent that can displace [^{3}H]-clonidine from bovine cerebral cortex membranes. This provides the evidence for the presence of 'clonidine-displacing substance' as described by Atlas and Burstein[1]. The CDS extracts are also capable of interacting with the non-adrenoceptor imidazoline binding sites on porcine renal cortex membranes labeled by [^{3}H]-idazoxan as previously reported on the properties of this substance[3]. This raises the possibility that CDS might be an endogenous ligand for both α₂-adrenoceptors and imidazoline receptors.

The binding of the extracts exhibited some selectivity for these 2 sites as previously reported[10]. The brain methanolic extracts, which were prepared in the same way as in this study, were less active for opiate receptors labeled by [^{3}H]-etorphine and inactive against α₁-adrenoceptors and muscarinic binding sites

labeled by [^{3}H]-prazosin and [^{3}H]-quinuclidinyl benzilate, respectively. In this study, the extract was approximately 2 folds more potent at α₂-adrenoceotors that at imidazoline receptors. However, this finding contrasts with that of Singh et al[10] which showed that the extract from bovine brain and lung were approximately 3-4 folds more active at the non-adrenoceptor imidazoline binding sites on rat kidney membranes labeled by [^{3}H]-idazoxan than at α₂-adrenoceptors on bovine cerebral cortex membranes labeled by [^{3}H]-clonidine. This discrepancy may be due to the difference in their ligand recognition properties of porcine and rat imidazoline sites on kidneys.

In the guinea-pig cerebral cortex slice model, the imidazoline derivative α₂-adrenoceptor agonist, UK14304, was used since it produced greater effect than clonidine which showed partially agonist activity. UK14304 (10^{-6} M) completely inhibited forskolin-stimulated cAMP accumulation.

This effect was mediated by α_2-adrenoceptors since it was prevented by the α_2-adrenoceptor antagonist, idazoxan. The inhibitory response to α_2-adrenoceptors activation in guinea-pig cortex slices was greater than that in the rat cerebral cortex slices. In the rat model, only 35% of the forskolin-stimulated cAMP accumulation was inhibited by UK14304 (10^{-6} M)[16]. Because of the greater degree of inhibition seen in the guinea-pig cortex, this tissue was used to examine whether CDS extracts possessed agonist activity at central α_2-adrenoceptors. This study found that methanolic CDS extract from bovine brain did not mimic the effects of α_2-adrenoceptor agonist, UK14304. The unexpected significant inhibitory effect of idazoxan in combination with the higher concentration of the brain methanolic extract on forskolin response might be due to some contaminants in the methanolic extract or idazoxan that might affect adenyl cyclase directly.

In the porcine palmar lateral vein, the vascular α_2-adrenoceptors has been demonstrated to be negatively coupled to cyclic AMP formation[17]. The degree of inhibition by α_2-adrenoceptor agonist, 10^{-7} M UK14304, on forskolin-stimulated [^{3}H]-cAMP accumulation in palmar lateral vein was found to be approximately 45% of the forskolin-induced response, which was comparable to that reported by Wright et al[17]. The inhibitory effect of α_2-adrenoceptor agonist, UK14304, on forskolin-stimulated response in porcine palmar lateral vein was also mediated by α_2-adrenoceptors, since the effect was prevented by idazoxan. However, the bovine CDS extract failed to mimic the effect of UK14304 or produce any significant potentiation of forskolin-stimulated [^{3}H]-cAMP accumulation. In addition, idazoxan did not alter the response of the brain extract on forskolin-stimulation.

The concentration of CDS extract used in functional assays was calculated to be in the range of that produced 50% inhibition of binding, however, much higher concentration might be needed since UK14304 also needed at least 10 folds of that concentration to produce functional effects.

In conclusion, no evidence was produced for the agonist activity of CDS extracts in both central and peripheral tissues in this study. No other evidence from any groups of investigators supports the CDS effects at α_2-adrenoceptors apart from the effect at prejunctional α_2-adrenoceptors of the rat vas deferens[11] and the proaggregatory action of the human platelets[12]. Although CDS extracts recognize α_2-adrenoceptors, the question arises whether the extracts possess any biological significance. The relation between binding characteristics and functional activities is still in doubt. In addition, different groups of investigators may determine different active compounds. Therefore, more highly purified extracts are essential in order to identify the active agents and to determine the biological activities of the same substance, and rule out the contaminants in the extract that may interfere with its functional activities.

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