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Short communication

Isolation of RP-HPLC pure clonidine-displacing substance from NG108-15 cells

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Abstract

A crude extract of clonidine-displacing substance (CDS) has previously been extracted from the NG108-15 cell line. This study aimed to purify CDS extracted from this cell line further, by the technique of reverse phase-HPLC (RP-HPLC), and subsequently determine whether this refined CDS bears any similarity to CDS's extracted from other tissues. Crude CDS was extracted from NG108-cells and fractionated by RP-HPLC eluting with a linear gradient of methanol (5–65%; 1 ml min^{-1} flow rate) over 50 min., and collected at 1 min. intervals. The pharmacological activities of the CDS fractions were determined by their abilities to displace bound [^{3}H]clonidine to α_2-adrenoceptors in rat brain membranes. RP-HPLC analysis of CDS revealed a pharmacologically active fraction distinct from agmatine, eluting at 24 min, corresponding to an absorbance peak observed at this time. Collectively, these results confirmed that CDS was present in the NG108-15 cell line. However, the RP-HPLC analysis showed the pharmacological activity to elute at a more hydrophobic gradient than previously observed with CDS's extracted from bovine tissues. These results support the notion of the existence of several CDS's.

Keywords: Clonidine-displacing substance; NG108-15 cell; Reverse phase HPLC; Imidazoline binding site

1. Introduction

The NG108-15 neuroblastoma × glioma hybrid cell line has been previously shown to express imidazole binding sites (Feinland et al., 1988) and the putative endogenous ligand for imidazoline binding sites, known as clonidine-displacing substance (CDS; Ernsberger et al., 1989).

Crude CDS has been extracted from several tissues and sera to date, including the bovine brain (Atlas and Burstein, 1984; Parker et al., 1999a), bovine lung (Singh et al., 1995; Parker et al., 1999a), bovine adrenal glands (Parker et al., 1999a), rat brain (Chan et al., 1997), rat serum, adrenal gland, gastric fundus and heart (Hensley et al., 1989; Meeley et al., 1992), human serum (Kreisberg et al., 1987; Dontenwill et al., 1993) and human cerebrospinal fluid (Goldberg-Stern et al., 1993). Currently, the chemical nature of CDS is undefined, although several substances have been postulated. Agmatine displays the ability to bind to imidazoline sites, but lacks the functionality associated with classical CDS, as defined by Atlas (1994). Prell et al. (1998) have also reported preliminary data suggesting that imidazoleacetic acid-ribotide is also an endogenous I_1-site ligand. Finally Grigg et al. (1998) have partially purified a CDS. Collectively, none of these compounds appear to resemble the classical CDS, based upon physiochemical characteristics, but these data illustrate the potential for several CDS molecules to exist endogenously.

Since CDS has been identified from many tissues and species, finding an optimum source from which to elucidate the structure of this substance is paramount. The aim of this particular study was therefore, to extract crude CDS from the NG108-15 cells and purify this CDS further via reverse phase-HPLC (RP-HPLC).

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2. Methods and materials

2.1. Culture of NG108-15 cells

NG108-15 cells were grown according to the methods described by Ernsberger et al. (1989). The cells were harvested by gently tapping the confluent layer into the media to form a suspension. Cells were then centrifuged at $2000 \times g$ for 2 min, and washed in phosphate buffered saline twice, before being stored immediately at -70°C.

2.2. Extraction of CDS from NG108-15 cells

CDS was extracted from the NG108-15 cells via the method of Ernsberger et al. (1989). Crude CDS was extracted from approximately 500×10^6 cells, passage 16 for the RP-HPLC analysis. The resulting crude methanolic CDS extracts were reconstituted into ultrapure water (1.4 ml) immediately prior to RP-HPLC analysis.

2.3. Rat whole brain membrane preparation

Rat whole brain membranes were prepared according to the methods of Mallard et al. (1992). For radioligand binding, membrane pellets were allowed to thaw to room temperature and washed twice by centrifugation, the membranes were subsequently resuspended in assay buffer to give 300–450 μg protein per assay tube. The protein content of the membrane preparations was determined by the method of Bradford (1976) using Coomassie blue with bovine serum albumin as the standard.

2.4. Radioligand binding studies

Radioligand binding studies were performed at 25°C in assay buffer (50 mM Tris–HCl, 1 mM $MgCl_2$, pH 7.4 at 4°C). Radioligand binding studies were performed to assess the ability of the NG108-15 CDS fractions at displacing [^{3}H]clonidine (3 nM) binding to α_2-adrenoceptors in rat whole brain membranes. Membrane aliquots, CDS extract and [^{3}H]clonidine were incubated in triplicate to equilibrium (30 min.) in a final volume of 0.5 ml. The specific binding component was determined by addition of rauwolscine (10 μM). Bound radioactivity was separated from free ligand by vacuum assisted rapid filtration through presoaked (0.5% polyethyleneimine) Whatman GF/B filters, using a Brandel M-24 cell harvester. Filters were washed twice with 3 ml of ice-cold assay buffer, and the trapped membrane bound radioactivity remaining on the filters was determined by liquid scintillation counting.

2.5. Reverse phase-high performance liquid chromatography (RP-HPLC)

The pharmacologically active crude methanolic CDS extract from NG108-15 cells was applied to a reverse phase column (RP-C18), and eluted using a linear gradient of methanol (5–65%, for 50 min.), in 0.05% trifluoroacetic acid, at room temperature at a flux rate of 1.0 ml min^{-1}. Ultraviolet (U.V.) absorption spectra at 276 nm was recorded. Fractions of 1.0 ml were collected, freeze-dried, reconstituted into ultrapure water (50 μl) and assayed for their pharmacological activities (see Section 2.4.).

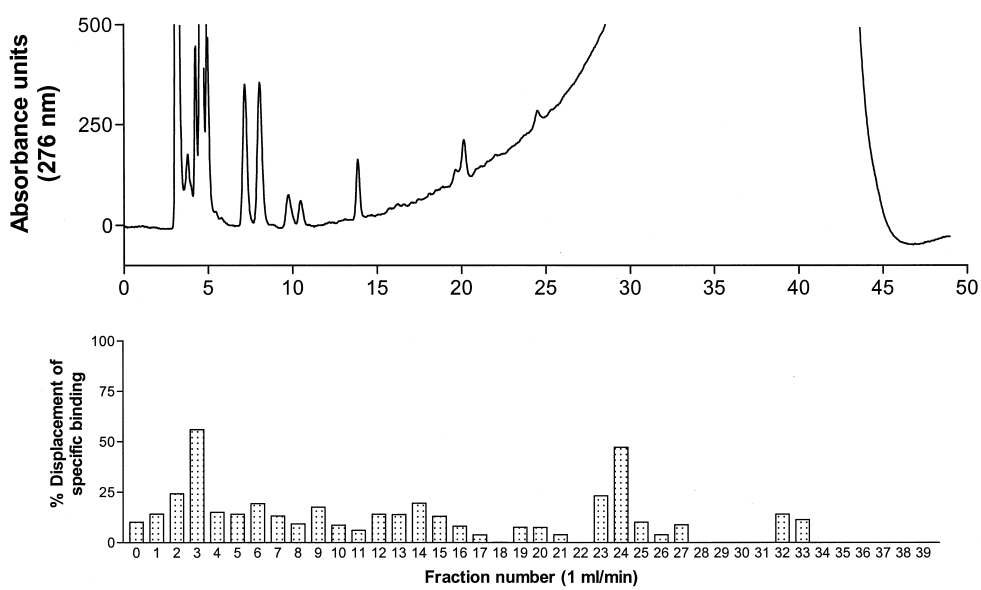

Fig. 1. Typical RP-HPLC trace for NG108-15 crude methanolic CDS extract (upper trace), with corresponding inhibition data for fractions collected at 1-min. intervals (lower trace). Fractions were assayed individually for their ability to displace [^{3}H]clonidine (3 nM) from binding to α_2-adrenoceptors present in rat brain membranes. Rauwolscine (10 μM) was used to define specific binding. Data represents the mean of 4 experiments.

2.6. Drugs and reagents

[^{3}H]Clonidine (specific activity = 65 Ci mmol^{-1}) stored at −20°C at a concentration of 1 mCi ml^{-1}, from NEN Life Science Products (UK). Rauwolscine from Sigma (UK). Dulbecco's Modified Eagle Medium and foetal calf serum from Gibco BRL (Life technologies, UK). All chemicals and reagents were of the highest analytical grade available.

3. Results

3.1. RP-HPLC analysis of CDS from NG108-15 cells

Fig. 1 shows a typical RP-HPLC trace of the crude CDS extracted from NG108-15 cells (500×10^6 in 1.4 ml ultrapure water) with corresponding binding inhibition data for 1-min fractions. The NG108-15 CDS extract exhibited a U.V. absorbance profile with a peak appearing at 21 min, similar to our earlier observations (Parker et al., 1999a). However, in contrast to our previous findings, this fraction did not exhibit the ability to displace bound radioligand to α_2-adrenoceptors, indicating that fraction 21 did not contain a CDS. This is in contrast to CDS extracted from bovine lungs, brain and adrenal glands which show potent activity in fraction 21 (Parker et al., 1999a). One unit of HPLC-pure CDS extracted from the NG108-15 cells has been defined as the amount required to displace 50% [^{3}H]clonidine bound to α_2-adrenoceptors present in rat whole brain membranes. In this respect, the fraction eluting at 24 min displaced over 45% of bound radioligand to α_2-adrenoceptors. This suggests that the fraction alone may contain approximately one unit of CDS.

In addition, there were other fractions collected from the RP-HPLC column which were eluted and found to displace [^{3}H]clonidine binding from rat brain membranes. The most prominent activity was found in a fraction eluting at 3 min which displaced over 50% of [^{3}H]clonidine bound to α_2-adrenoceptors. Subsequently, a series of standards were eluted from the column and the endogenous compounds, agmatine, histamine and noradrenaline were all shown to elute at 3 min under the conditions used in this study (Table 1).

Table 1
Elution times of various compounds from the RP-HPLC column, using a methanol gradient (5–65%; 50 min) in 0.05% trifluoroacetic acid, at room temperature and a flux rate of 1 ml min^{-1}. Absorption at 276 nm was recorded

Compound (1 mg ml^{-1})	Elution time (in min.; 5–65% methanol)
Agmatine	3.0
Histamine	3.0
Noradrenaline	3.0
Clonidine	17.0
Rilmenidine	21.5

4. Discussion

This study has supported previous work indicating the presence of a form of CDS in an extract from the neuroblastoma × glioma hybrid cell line (NG108-15 cells). Ernsberger et al. (1989) isolated crude CDS from NG108-15 cells, and showed that this crude extract displaced [^{3}H] *para*-aminoclonidine bound to both α_2-adrenoceptors and I_1-sites in bovine frontal cortex membranes. Previously, we have reported that the crude extract of CDS isolated from the NG108-15 cells displaced radioligand bound from not only rat brain α_2-adrenoceptors, but also from I_1- and I_2-sites in rat kidney and rat brain membranes, respectively (Parker et al., 1999b). Furthermore, our study showed that 10 fold and 5 fold less CDS was required to displace 50% of bound radioligand from I_1- and I_2-sites, respectively, relative to 1 unit required to displace 50% of [^{3}H]clonidine from α_2-adrenoceptors (Parker et al., 1999b). Collectively, these data indicate that imidazoline binding sites may possess a higher affinity for CDS than the α_2-adrenoceptors. However, the present study demonstrates the need to purify CDS. It is difficult to interpret the total activity of the crude CDS into what truly represents the pharmacological components that were present in fraction 24.

The reverse phase-HPLC analysis of the crude methanolic CDS extracted in this study has revealed novel data. Previously, we have shown that partially purified ''classical'' CDS (as defined by Atlas, 1994) eluted from the RP-HPLC column exhibiting three peaks of U.V. absorbance, with the pharmacological activity eluting with the second peak, at 20–21 min (Parker et al., 1999a). Under similar conditions, the CDS extracted from NG108-15 cells exhibited only a single peak around this time, corresponding to the second peak of classical CDS eluting at 21 min. However, this fraction collected at 21 min, exhibited minimal pharmacological activity. Furthermore, the fraction eluting at 24 min (which relates to the peak eluting at this time, and is unassociated with the profile of Atlas, 1994) exhibited over 45% displacement of radioligand bound to α_2-adrenoceptors.

The fraction eluting at 3 min from the RP-HPLC column was shown to displace over 50% of radioligand bound to α_2-adrenoceptors. Under our experimental conditions, the previously proposed endogenous ligand for imidazoline binding sites, agmatine (Li et al., 1994), also eluted at 3 min from the RP-HPLC column. Other endogenous ligands, histamine and noradrenaline were also shown to elute at this time point from the column in our study, which may explain, to a degree, the pharmacological activity observed in this fraction. Clearly the CDS eluting at 24 min from the RP-HPLC column was distinct from agmatine.

It is concluded that CDS extracted from NG108-15 cells possesses different components than ''classical'' CDS obtained from tissues, using similar extraction procedures.

NG108-15 cells do not possess the inactive compounds found within the bovine lung, brain or adrenal glands, which give rise to the inactive first and third peaks of the ''classical'' CDS profile (Parker et al., 1999a). Moreover, the present study shows that the CDS extracted from the NG108-15 cells is a more hydrophobic CDS than that isolated from bovine lung, brain and adrenal glands, since it elutes from the column somewhat later than the previously proposed endogenous ligand for imidazoline binding sites, agmatine (Li et al., 1994), and the CDS found in other tissues (Parker et al., 1999a). Thus it can be suggested that the CDS eluting from the RP-HPLC column, in this study, differs chemically from the CDS extracted from bovine tissues, and as such further supports the notion of several CDS's. The potent activity contained within the 24-min fraction of the NG108-15 CDS requires further investigation.

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References

Atlas, D., 1994. Identifying clonidine-displacing substance. Science 266, 462–463.

Atlas, D., Burstein, Y., 1984. Isolation and partial purification of a clonidine-displacing endogenous brain substance. Eur. J. Pharmacol. 144, 287–293.

Bradford, M.M., 1976. A rapid sensitive method for the quantification of protein using the principle of protein dye binding. Anal. Biochem. 72, 248–252.

Chan, S.L.F., Atlas, D., James, R.F.L., Morgan, N.G., 1997. The effect of the putative endogenous imidazoline receptor ligand, clonidine-displacing substance, on insulin secretion from rat and human islets of Langerhans. Br. J. Pharmacol. 120, 926–932.

Dontenwill, M., Molines, A., Verdun, A., Bricca, G., Laurent, S., Bousquet, P., 1993. A circulating substance cross-reacting with antiimidazoline antibodies. Detection in serum in relation to hypertension. J. Clin. Invest. 92, 1068–1072.

Ernsberger, P., Meeley, M.P., Reis, D.J., 1989. Neuroblastoma-glioma hybrid cells contain clonidine-displacing substance. Eur. J. Pharmacol. 174, 135–138.

Feinland, G., Ernsberger, P., Meeley, M.P., Evinger, M.J., Reis, D.J., 1988. Differential expression of imidazole and alpha$_2$-adrenergic receptors and clonidine-displacing substance in NG108-15, glial, and chromaffin cells. Soc. Neurosci. Abstr. 14, 412.

Goldberg-Stern, H., Atlas, D., Schwartz, L., Achiron, A., Ziv, I., Djaldetti, R., Zoldan, Y., Melamed, E., 1993. Detection and measurement of an endogenous clonidine-displacing substance in human cerebrospinal fluid. Brain Res. 601, 325–328.

Grigg, M., Musgrave, I.F., Barrow, C.J., 1998. Isolation and partial structure determination of a clonidine-displacing substance from bovine lung and brain. J. Auton. Nerv. Syst. 72, 86–93.

Hensley, M.L., Meeley, M.P., McCauley, P.M., Ernsberger, P., Reis, D.J., 1989. Clonidine-displacing substance is present in peripheral tissues of the rat. Am. J. Hypertens. 2, 917–919.

Kreisberg, G.A., Diamant, S., Diamant, Y.Z., Atlas, D., 1987. Raised levels of an endogenous nonadrenergic substance in the serum of pregnancy-induced hypertension patients. Isr. J. Med. Sci. 23, 1194–1197.

Li, G., Regunathan, S., Barrow, C.J., Eshraghi, J., Cooper, R., Reis, D.J., 1994. Agmatine: an endogenous clonidine-displacing substance in the brain. Science 263, 966–969.

Mallard, N.J., Hudson, A.L., Nutt, D.J., 1992. Characterization an autoradiographical localization of non-adrenoceptor idazoxan binding sites in the rat brain. Br. J. Pharmacol. 106, 1019–1027.

Meeley, M.P., Hensley, M.L., Ernsberger, P., Felsen, D., Reis, D.J., 1992. Evidence for a bioactive clonidine-displacing substance in peripheral tissues and serum. Biochem. Pharmacol. 44, 733–740.

Parker, C.A., Hudson, A.L., Nutt, D.J., Dillon, M.P., Eglen, R.M., Crosby, J., 1999a. Comparison of crude methanolic CDS extracts from various tissues. Ann. N.Y. Acad. Sci. 881, 92–96.

Parker, C.A., Hudson, A.L., Nutt, D.J., Dillon, M.P., Eglen, R.M., Crosby, J., 1999b. Reverse-phase-HPLC analysis of clonidine-displacing substance present in NG108-15 cells. Br. J. Pharmacol. 128, 194 pp.

Prell, G.D., Martinelli, G.P., Ernsberger, P., 1998. Imidazoleacetic acid-ribotide, an endogenous imidazoline I1 receptor ligand. Soc. Neurosci. Abstr. 24, 246.18.

Singh, G., Hussain, J.F., MacKinnon, A., Brown, C.M., Kendall, D.A., Wilson, V.G., 1995. Evidence for the presence of a non-catecholamine, clonidine-displacing substance in crude, methanolic extracts of bovine brain and lung. Naunyn-Schmiedberg's Arch. Pharmacol. 351, 17–26.