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Short communication

MUSCARINE BINDING SITES IN BOVINE ADRENAL MEDULLA

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The presence of muscarinic binding sites in the bovine adrenal medulla was investigated using [^{3}H]QNB and the bovine adrenal medulla. Scatchard analysis combined with computer analysis yielded data consistent with a two binding site configuration. K_Ds of 0.15 and 14 nM and B_{max}s of 29 and 210 fmol/mg protein, respectively, were observed. Displacement of [^{3}H]QNB by various cholinergic agents is, in order of decreasing potency: QNB, dexetimide, atropine, scopolamine, imipramine, desipramine, oxotremorine, pilocarpine, acetylcholine, methacholine and carbachol. These results demonstrate the presence of more than one muscarine binding site in the bovine adrenal gland.

Muscarine binding sites Bovine adrenal medulla QNB

1. Introduction

Catecholamine secretion from the adrenal medulla is mediated primarily by nicotinic cholinergic receptors. The ability of muscarinic cholinergic agonists to stimulate catecholamine secretion from the adrenal medulla appears to be species dependent. Pilocarpine can stimulate catecholamine secretion in the rat adrenal gland (Wakade and Wakade, 1983) but not in cattle (Wilson and Kirshner, 1977). However, a binding site for muscarinic agents has been reported in the bovine adrenal medulla (Kayaalp and Neff, 1979). The specific role of muscarine receptor activation in the bovine adrenal medulla is unclear. Recent evidence indicates that an increase in cytosolic Ca^{2+} independent of extracellular Ca^{2+} can be elicited by muscarine receptor agonists and that this increase is subthreshold for catecholamine secretion in bovine chromaffin cells (Kao and Schneider, 1985). We have shown an effect of atropine in inhibiting the acetylcholine stimulation of norepinephrine, but not epinephrine, secretion from perfused beef adrenal glands (Barron and Hexum, 1983). Muscarine receptor agonists have also been implicated in regulating phosphatidylinositol labelling (Adnan and Hawthorne, 1981) and cGMP levels (Yanagihara et al., 1979) in bovine adrenal medullary cells. Based on these observations we felt that a more extensive analysis of the muscarine binding site was warranted. We decided to reinvestigate the muscarine binding site in the bovine adrenal medulla using the specific muscarine receptor antagonist [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB).

2. Materials and methods

2.1. Purified chromaffin cell membrane fraction

Preparation of a partially purified chromaffin cell membrane fraction was done by differential centrifugation. Adrenal medullary tissue was

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minced, and homogenized with a Polytron followed by a Teflon postle in glass with 5 volumes of 0.32 M sucrose. The homogenate was centrifuged at $400 \times g$ for 20 min and the pellet (P_1) discarded. The supernatant was centrifuged at $8700 \times g$ for 20 min, the pellet (P_2) was resuspended in 5 volumes of 0.32 M sucrose and centrifuged again at $8700 \times g$ for 20 min. The two $8700 \times g$ supernatants (S_2) were combined and centrifuged at $81\,000 \times g$ for 60 min to produce the final pellet (P_3 membrane fraction) and final supernatant (S_3). The final pellets (P_2 and P_3) were weighed and resuspended in 10 volumes of 0.32 M sucrose, the final supernatant (S_3) was used as decanted from the P_3 fraction. The supernatant (S_3), final pellet (P_3), and the previous pellet (P_2) were used to assay for [^{3}H]QNB specific binding as described below. The P_3 fraction specifically bound significantly more [^{3}H]QNB than either the P_2 or S_2 fraction and binding was linear with increasing protein concentration up to 60 μg protein/ml. [^{3}H]QNB binding was not significantly different when either fresh or frozen membrane preparations were used. Therefore P_3 membranes, frozen at -80°C, were used in the binding studies described here.

2.2. Radioreceptor binding assay

Aliquots of the P_3 membrane preparation (120 μg protein) were incubated with [^{3}H]QNB (0.05-30 nM) in 2.0 ml of 0.05 M Na/K phosphate buffer, pH 7.4, for 60 min at 37°C plus or minus 5 μM atropine to determine non-specific binding. The incubation was stopped by rapid filtering onto Whatman GF/B glass fiber filters using a Brandel cell harvester (Gaithersurg, Md) and washing three times with 5.0 ml of cold buffer. All determinations were carried out in triplicate.

Displacement of 0.7 nM [^{3}H]QNB by various drugs was investigated with or without 5 μM atropine as the indicator of non-specific binding. Physostigmine (10 μM), which had no effect on QNB binding, was included when the binding of acetylcholine was measured. The IC_{50} values were calculated from the graph of log drug concentration versus logit (%) after linear regression analysis [logit (%) = ln (% bound)/(100 − % bound)]. Data from [^{3}H]QNB saturation binding experiments were analyzed by Scatchard plot. The Scatchard analysis was confirmed by computer analysis using the non-linear curve fitting program, LIGAND.

2.3. Materials

[^{3}H]QNB was purchased from New England Nuclear. Atropine, pilocarpine, oxotremorine, scopolamine, acetylcholine, methacholine, carbachol, imipramine, desipramine and physostigmine were purchased from Sigma Chemical Co. Dexetimide was generously provided by Janssen Pharmaceutica. Unlabeled QNB was generously provided by Hoffman-La Roche, Inc.

3. Results

3.1. Saturation of [^{3}H]QNB binding

Using the frozen P_3 membrane preparation, increasing concentrations of [^{3}H]QNB (0.05-30 nM) were added with or without 5 μM atropine. The specific binding of [^{3}H]QNB to the P_3 membranes displayed saturation of binding to the site(s) with two plateaus between 0.1 and 5 nM and then rapidly increased up to 30 nM (fig. 1A).

Scatchard analysis of the [^{3}H]QNB binding to the P_3 membrane fraction revealed a 2-site configuration (fig. 1B). Linear regression analysis was combined with Scatchard analysis and confirmed by computer analysis using the LIGAND curve fitting program. K_Ds of 0.15 and 14 nM and B_{max}s of 29 and 210 fmol/mg protein were thereby determined.

3.2. [^{3}H]QNB displacement by cholinergic agents

The displacement of [^{3}H]QNB from the P_3 membrane fraction was investigated by incubation of 0.7 nM [^{3}H]QNB with increasing concentrations of various drugs that possess cholinergic properties, with and without 5 μM atropine as the indicator of nonspecific binding. The antagonists QNB, dexetimide, atropine, and scopolamine were most potent in displacing the [^{3}H]QNB. The agonists oxotremorine, acetylcholine, pilocarpine,

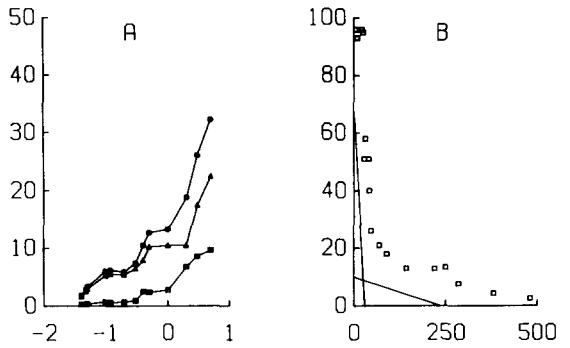

Fig. 1. (A) The P_3 membrane preparation was suspended in 10 volumes of 0.32 M sucrose and a 20 μl aliquot was used in the binding assay with 0.05-30 nM [^{3}H]QNB with or without 5 μM atropine to determine specific binding. Each point is the average of four determinations. Total binding (●———●). Specific binding (▲———▲). Non-specific binding (■———■). Ordinate: fmol bound; abscissa: log [^{3}H]QNB (nM). (B) Data from [^{3}H]QNB saturation experiments (specific binding) was analyzed by Scatchard plot (represented by open squares), subjected to linear regression analysis and the K_D and B_{max} determined graphically. Results from Scatchard analysis were confirmed using LIGAND (represented by lines). Ordinate: bound/free$\times 10^6$; abscissa: bound (fmol/mg protein).

methacholine and carbachol were very weak displacers while the antidepressants, imipramine and desipramine, which have prominent anticholinergic effects, were intermediate in displacement strength (table 1).

TABLE 1

[^{3}H]QNB displacement from bovine adrenal medulla membranes. Data from drug competition curves were subjected to linear regression analysis. The IC_{50} values were calculated from the graph of log drug concentration versus logit (%): logit (%) = ln [(% bound)/(100 − % bound)].

Drug	IC_{50} (μM)
QNB	0.009
Dexetimide	0.05
Atropine	0.06
Scopolamine	0.157
Imipramine	1.1
Desipramine	2.8
Oxotremorine	28.5
Pilocarpine	350.0
Acetylcholine	560.0
Methacholine	> 4000.0
Carbachol	> 5000.0

4. Discussion

Muscarine receptors in the rat adrenal medulla mediate catecholamine secretion (Wakade and Wakade, 1983). However, muscarine receptor agonists apparently do not stimulate catecholamine secretion in bovine adrenal glands (Wilson and Kirshner, 1977) but muscarinic binding has been identified in bovine adrenal glands (Kayaalp and Neff, 1979). Recently, muscarinic modulation of catecholamine release from the bovine adrenal medulla has been demonstrated (Barron and Hexum, 1983).

The activity of the muscarine receptor in stimulus-secretion coupling requires further study to determine the mechanism by which the muscarine receptor participates and which subtype of muscarine binding sites may be involved. A small increase in intracellular Ca^{2+} which was independent of extracellular Ca^{2+} was produced by muscarine receptor activation in bovine chromaffin cells (Kao and Schneider, 1985). An increase in intracellular Ca^{2+} is necessary for stimulus-secretion coupling leading to exocytosis of catecholamines. The increase in intracellular Ca^{2+} stimulated by muscarinic agents (Kao and Schneider, 1985) was below the threshold for catecholamine secretion but may be enough for muscarinic modulation of the larger nicotinic stimulated Ca^{2+} influx leading to catecholamine secretion.

Studies on the muscarine binding site in other systems (heart, brain and myenteric plexus) have shown a multiplicity of muscarine binding site subtypes (Burgen, 1984). The possibility of more than one muscarine binding site subtype being present in the bovine adrenal medulla requires further investigation. The action of muscarinic agents on different cell functions [i.e., cGMP levels (Yanagihara et al., 1979), phosphatidylinositol labelling (Adnan and Hawthorne, 1981), catecholamine secretion, intracellular Ca^{2+} levels (Kao and Schneider 1985)] suggests the possibility that more than one subtype of muscarine binding site will be found in the bovine adrenal medulla.

The presence of a muscarine binding site in the bovine adrenal medulla as evidenced by the specific binding of [^{3}H]QNB to a chromaffin cell mem-

brane preparation was also demonstrated by Kayaalp and Neff (1979). However, there are differences between the data reported by Kayaalp and Neff and the data reported here. The K_D reported by Kayaalp and Neff was 0.07 nM with a B_{max} of 3 fmol/mg protein, whereas we found K_Ds of 0.15 and 14 nM with B_{max}s of 29 and 210 fmol/mg protein. These differences may be explained by considering several factors. Kayaalp and Neff used only six concentrations of [^{3}H]QNB (0.018-0.54 nM) for their binding study and Scatchard analysis. This range of [^{3}H]QNB concentrations was insufficient to distinguish a second site with a K_D greater than 0.54 nM. The binding studies presented here used [^{3}H]QNB (0.05-30 nM) with at least 12 concentrations to give good definition to the Scatchard analysis. From this analysis, data consistent with two binding sites were found.

The use of different chromaffin cell membrane preparations is another factor which may explain the disparity in results. Kayaalp and Neff (1979) used the P_2 fraction after centrifugation of the adrenal medulla homogenate. We compared the binding of [^{3}H]QNB to the different centrifugation fractions. The P_3 fraction bound considerably more [^{3}H]QNB than the P_2 membrane fraction using equivalent protein concentrations (data not shown).

Another factor which may explain the disparity in results is the agent used to determine the non-specific binding. Kayaalp and Neff (1979) used oxotremorine (200 μM) to determine non-specific binding. Oxotremorine is a much weaker displacer of [^{3}H]QNB binding than atropine (table 1). Atropine was used to determine non-specific binding in this study (5 μM). Both QNB and atropine are muscarine receptor antagonists. Oxotremorine being a strong agonist would be expected to have different binding characteristics from the antagonists (Burgen, 1984).

Any or all of these reasons may partially explain the disparity seen in the results presented here and the results of Kayaalp and Neff. Jumblatt and Tishchler (1982) reported a [^{3}H]QNB binding site in rat pheochromocytoma cell line (PC 12). Pheochromocytoma cells are known to be derived from the adrenal medulla. The values they reported ($K_D = 0.146$ nM, $B_{max} = 32.7$ fmol/mg protein) agree with the values for the high affinity site in this study.

[^{3}H]QNB binding to the chromaffin cell membrane preparation is linear with respect to protein content and saturable with two plateaus at 0.1 and 1 nM. Enrichment of [^{3}H]QNB binding to the P_3 membrane preparation as compared to the other cell fractions suggests that the binding site is on the chromaffin cell membrane. The [^{3}H]QNB binding site reported here is different from the muscarine binding sites characterized in brain, heart and gastrointestinal tract in K_D, B_{max} and potency of displacing drugs. The IC_{50} values for the ten drugs reported here are on the order of 19-200 fold less efficacious in displacing [^{3}H]QNB than is seen in other tissue studies (Ishibe et al., 1985; Ravikumar and Sastry, 1985). The displacement of the [^{3}H]QNB binding by cholinergic agents is greater with antagonists than with agonists, as is expected. The muscarinic binding in the bovine adrenal medulla confirmed by these studies indicates there may be a muscarinic component in the control of catecholamine release. Further study is needed to define the extent of muscarinic involvement in the bovine adrenal medulla catecholamine release process.

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