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SIMULTANEOUS ASSAY OF MUSCARINIC AND β-ADRENERGIC RECEPTORS USING A DOUBLE ISOTOPE TECHNIQUE

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Summary. Muscarinic receptor and β-adrenergic receptor binding were measured simultaneously in a membrane fraction of bovine tracheal smooth muscle using [^{3}H]-L-quinuclidinyl benzilate and [^{125}I]-(-)iodocyanopindolol. The binding characteristics, affinity and receptor density, obtained in the double receptor assay and in the control experiments were the same within experimental error. Moreover, there appears to be neither a significant influence of an excess of d,l-propranolol on [^{3}H]-L-quinuclidinyl benzilate binding nor a significant influence of an excess of l-quinuclidinyl benzilate on [^{125}I]-(-)iodocyanopindolol binding. The method is advantageous where both receptors have to be assayed and where limited amounts of biological material, like in biopsy specimen, are available.

With the availability of tritium and iodine-125 labeled receptor ligands, which can be counted differentially, simultaneous determination of two types of receptors in one sample is possible. A similar double receptor assay has been used recently for measuring estrogen and progesterone receptors in human breast tumor cytosol (1).

We here report the simultaneous determination of β-adrenergic and muscarinic receptors in bovine tracheal smooth muscle. The presence of these receptors in this tissue has been demonstrated before (2, 3).

MATERIALS AND METHODS

^{3}H-QNB (33.2 Ci/mmol) and ^{125}I-CYP (2200 Ci/mmol) were purchased from NEN-Europe (Doorn, The Netherlands), d,l-propranolol hydrochloride was obtained from Sigma Chemical Co., St. Louis, USA. l-QNB was a gift from Dr. G. Lambrecht, Johann Wolfgang Goethe-Universität, Frankfurt/M, West Germany. Other reagents were of the highest quality available commercially.

The abbreviations used are: ^{3}H-QNB: quinuclidinyl benzilate, L - [benzilic-4,4'-^{3}H(N)] ; ^{125}I-CYP: (-)-iodocyanopindolol, [^{125}I]; l-QNB: R(-)-quinuclidinyl benzilate; LSC: liquid scintillation counter.

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Bovine tracheal smooth muscle was prepared and homogenized with carborundum as described previously (2). The resulting 100,000 g membrane pellets were stored at -20°C and resuspended before use in Krebs-Henseleit buffer to give a final tissue concentration of about 20 mg original wet weight/ml (0.2 mg protein/ml). The method used for the assay of particle bound receptor was the rapid centrifugation method as described previously (4), except that the samples were incubated during 90 minutes at 37°C. At the end of the centrifugation step 0.5 ml supernatant was taken for assaying free concentration of radioactive ligand. Then the remaining supernatant was removed by suction and the tips of the centrifugation tubes were cut off for assaying bound concentration of radioactive ligand in the pellet. In the single ^{3}H-QNB assay 0.5 ml supernatant was mixed with 10 ml Instagel (Packard). The pellets were dissolved in 1 ml Soluene -100 (Packard) and mixed with 15 ml of Instagel/1 N HCl(9:1). The samples were counted in the tritium channel of a Packard Tricarb 460 LSC. In the single ^{125}I-CYP assay radioactivities in 0.5 ml supernatant and in the pellet were counted directly on a Nuclear Enterprises 1612 γ-counter. In the double receptor assay supernatants and pellets were first counted in the γ-counter, then scintillation cocktail was added as described for the single ^{3}H-QNB assay, followed by counting in the tritium channel of the LSC. Protein concentrations were determined by the method of Lowry as modified by Peterson (5) using bovine serum albumin as standard. The experimental data were analysed and binding parameters calculated by employing a general non-linear, least squares curve fitting routine using the Gauss-Newton algorithm (cf 6).

RESULTS AND DISCUSSION

Detection of iodine-125 can be performed by γ-counting (35.5 KeV γ-ray and 27.5 KeV X-ray) with a 78% efficiency and by liquid scintillation counting (conversion and Auger electrons at 12 and 40 KeV and 3.5 KeV X-rays) with an efficiency of 76% in an unquenched emulsifier scintillator solution (7). Differential counting of tritium and iodine-125 in one sample can be achieved by liquid scintillation counting in two spectrum regions as recommended in the Packard 460C instruction manual (8). As both isotopes contribute to counts accumulated in each spectrum region, under certain circumstances (i.e. if large iodine-125/tritium count rate ratios are involved) relatively large errors in the tritium count rates will occur. This can be avoided by measuring iodine-125 in a γ-counter first. With the counting efficiencies of iodine-125 in the γ-counter and in the tritium channel of the LSC known, the contribution of iodine-125 to the count rate in the tritium channel of the LSC can be calculated. Tritium count rate follows from subtracting the contribution of iodine-125 from total count rate in the tritium channel. The reliability of the method has been tested by correlating tritium count rates in the absence and in the presence of a sevenfold excess of iodine-125. ($y = 1.02\ x + 2.9$; $r = 0.9999$; $n = 9$). To avoid too high count rate ratios

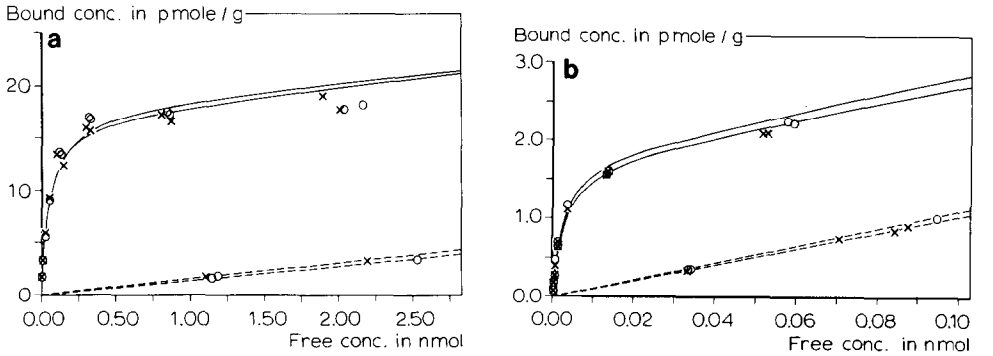

Figure: 1a) Total (–) and non-specific (--) binding of ^{3}H-QNB to bovine tracheal smooth muscle in the single (x) and double (o) assay.

1b) Total (–) and non-specific (--) binding of ^{125}I-CYP to bovine tracheal smooth muscle in the single (x) and double (o) assay.

in the double receptor assay the ^{3}H-QNB concentration was increased parallel with the ^{125}I-CYP concentration.

In a set of three experiments with the same tissue homogenate, the concentration dependent binding of ^{3}H-QNB alone, of ^{125}I-CYP alone and of ^{3}H-QNB + ^{125}I-CYP combined, was measured. Non-specific binding of ^{3}H-QNB and ^{125}I-CYP in the single experiments was determined in the presence of 0.1 µM l-QNB or 1 µM d,l-propranolol respectively. In the combined experiment non-specific binding of ^{3}H-QNB and ^{125}I-CYP was determined simultaneously in the presence of 0.1 µM l-QNB + 1 µM d,l-propranolol. The results of these experiments are given in fig. 1. No significant influence of ^{125}I-CYP on ^{3}H-QNB binding and vice versa can be discerned. The binding parameters of these experiments are summarized in table 1. In a second set of experiments carried out with another membrane suspension,total and non-specific binding of ^{3}H-QNB in the absence and presence of an excess of 1 µM d,l-propranolol was measured and in an analogous way total and non-specific binding of ^{125}I-CYP in the absence and presence of an excess of 0.1 µM l-QNB was determined. The binding parameters of these experiments are summarized in table 1. No significant influence of an excess of d,l-propranolol on ^{3}H-QNB binding or of l-QNB on ^{125}I-CYP binding was found. A simultaneous assay of muscarinic and β-adrenergic receptors in one sample is feasible without complications. The double receptor assay may equally well be applied to the assay of any pair of receptors when different-

Table 1. Binding parameters of ^{3}H-QNB and ^{125}I-CYP in bovine tracheal smooth muscle

Type of experiment	K_D(nM)	R_T(pmol/g)	S_Y(ml/g)
A. ^{3}H-QNB (single receptor assay)	0.055 (6%)	17.3 (3%)	1.5
^{3}H-QNB (double receptor assay)	0.063 (7%)	18.0 (4%)	1.4
^{125}I-CYP (single receptor assay)	0.0026 (6%)	1.7 (4%)	10.2
^{125}I-CYP (double receptor assay)	0.0025 (8%)	1.8 (6%)	10.9
B. ^{3}H-QNB	0.058 (8%)	16.7 (4%)	1.2
^{3}H-QNB + 1.0 µM d,l-propranolol	0.057 (5%)	16.9 (3%)	1.2
C. ^{125}I-CYP	0.0028 (5%)	1.4 (3%)	9.4
^{125}I-CYP + 0.1 µM l-QNB	0.0021 (6%)	1.4 (4%)	9.9

K_D is the dissociation constant in nM, R_T is the binding capacity in pmol/g original wet weight tissue. S_Y is the coefficient for non-specific binding i.e. by multiplying its value with the free concentration of ligand, the non-specific binding in pmol/g original wet weight tissue at that concentration is obtained. Values in parentheses indicate relative standard errors. The sets of experiments indicated with A, B and C are performed with different tissue homogenates. 1 g original wet tissue corresponds with 10 mg protein in the final homogenates.

ially labeled ligands are available, i.e. α_1- and β-adrenergic receptors using [^{3}H]-prazosin as α_1-selective ligand and ^{125}I-CYP as β-adrenergic ligand; muscarinic and α_1-adrenergic receptors using ^{3}H-QNB and [^{125}I]-2[β-(4-hydroxy-3-iodophenyl)ethyl aminomethyl] tetralone. For steroid receptors the double receptor assay has recently been applied to the simultaneous determination of estrogen and progesterone receptors in tumor cytosols using [^{125}I]-estradiol and [^{3}H]-R5020 (1). In case receptor density and affinity ratios are unfavourable, the specific activity of one of the radioligands may be lowered by diluting with unlabeled ligand to optimalize the count rate ratio of iodine-125/tritium in the sample.

We are currently involved in measuring muscarinic and β-adrenergic receptors in lung tissue from patients with chronic obstructive lung diseases. These studies are often hampered by limited amounts of tissue. With the double receptor assay,tissue and time can be saved. Moreover, the double receptor assay might be especially advantageous to study a functional relationship between different types of receptors.

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