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THE RELATIONSHIP BETWEEN MUSCARINIC RECEPTOR BINDING AND ION MOVEMENTS IN RAT PAROTID CELLS

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SUMMARY

1. The relationship of muscarinic receptor binding to ion fluxes induced by muscarinic agonists was investigated in rat parotid cells.

2. Receptor binding was measured with [^{3}H]quinuclidinyl benzilate in living, dispersed parotid acinar cells. These same cells were used to determine concentration–effect relationships (ion fluxes) for agonists.

3. Receptor binding of antagonists accurately reflected the pharmacologically determined affinities. In the case of agonists, apparent pharmacological affinities were greater than binding affinities by a factor of 12·5 for methacholine and 18·7 for carbachol.

4. The reason for the discrepancy between agonist binding and effect is not known with certainty, but the existence of 'spare' receptors is considered a possibility.

5. The total number of muscarinic receptors is estimated to be 23,000/cell. If some are spare, as few as 1800/cell may suffice to fully activate the available Ca channels.

INTRODUCTION

In recent years, the use of specific radiolabelled ligands has greatly facilitated the study of cell membrane receptors by permitting biochemical determinations of receptor number and binding kinetics (Birdsall & Hulme, 1976; Furchgott, 1978). The muscarinic receptor ligand, [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) has proven especially useful in studies of muscarinic receptors in the C.N.S. and periphery (Snyder, Chang, Kuhar & Yamamura, 1975; Birdsall & Hulme, 1976). Thus, the quantitative relationship between receptor occupancy and biological response can be directly examined.

The rat parotid gland is an excellent model for such an evaluation. The non-excitable nature of the cell membrane (Roberts & Petersen, 1978) has the consequence that ion channels are probably activated either by second messengers or by direct receptor actions. Further, the initial ion movement (Ca influx) as well as secondary responses (K and Na movements) can be readily measured (Putney, 1978; Putney, Van De Walle & Leslie, 1978). Finally, by using enzymically dispersed isolated

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acinar cells, receptor binding measurements can be made on the same physiological preparations employed for ion flux measurements (Strittmatter, Davis & Lefkowitz, 1977*a*, *b*).

This report describes an attempt to relate, by concentration–effect relationships, the binding of agonists to muscarinic receptors, Ca influx, and Ca-activated K efflux in rat parotid acinar cells.

METHODS

Dispersed parotid cells were prepared by the methods described previously (Putney *et al.* 1978; Kanagasuntheram & Randle, 1976). Cells were resuspended and all experiments carried out in physiological Ringer solution (for composition see Putney *et al.* 1978) at 37 °C without bovine serum albumin. In preliminary experiments, it was found that the specific binding of [^{3}H]QNB was inhibited about 35% by 2% bovine serum albumin, presumably due to binding of [^{3}H]QNB to the albumin. Since the cells maintained appropriate ion gradients (data not shown) and responded appropriately (see below) in the absence of albumin, the albumin was not included in the final incubation medium for subsequent experiments.

For ligand binding measurements, cells were transferred into 5 ml. plastic vials, 2·5 ml. per vial, and pre-incubated for 30 min. Subsequently, [^{3}H]QNB was added to the suspension and at various times (usually 30 min, see Results) duplicate aliquots of the suspension were transferred to large volumes (usually 10 ml.) of ice-cold sucrose (320 mM). The suspensions were centrifuged, the pellets rinsed once with cold sucrose, and resuspended and lysed as described previously (Putney *et al.* 1978). Protein and ^{3}H in the pellets were determined as described (Putney *et al.* 1978). The concentration of cells in each vial was not sufficient to significantly reduce the [^{3}H]QNB concentration in the medium. The estimated receptor concentration was less than one hundredth the estimated dissociation constant (K_D) for [^{3}H]QNB binding (Chang, Jacobs & Cuatrecasas, 1975).

The total time required to centrifuge the cells by this procedure was generally less than 2 min. To ensure that no labelled material dissociated from the cells in the cold sucrose solution, preliminary experiments were carried out wherein the centrifugation was delayed 1, 2 and 4 min after dilution of the cells in cold sucrose. The decrease in binding over this period was not statistically detectable, and must have been less than 5% of the initial value, indicating that very little dissociation of the [^{3}H]QNB was occurring during the cell separation procedure.

Specific binding of [^{3}H]QNB at various concentrations (0·05–10·0 nM) was determined by using two vials of cells for each concentration of [^{3}H]QNB, one vial containing [^{3}H]QNB plus a high concentration of unlabelled drug to measure non-specific binding, the other vial containing [^{3}H]QNB only to measure total binding. The difference between the amount of [^{3}H]QNB bound in the two vials is termed specific binding. Data are expressed as f-mole [^{3}H]QNB bound per mg protein.

The ability of muscarinic agonists and antagonists to inhibit [^{3}H]QNB binding was similarly investigated. A known concentration of [^{3}H]QNB (0·5 nM) was added to each vial in the presence of various concentrations of drugs. Data were expressed as per cent inhibition of total [^{3}H]QNB binding.

The concentration–effect relationships for K release due to muscarinic agents were determined by incubating cells with agonist (or agonist and antagonist) and 1 mM ouabain for 2 min. Ouabain was used to inhibit reuptake of K from the medium. Cells were separated in cold sucrose, and K content of the pellet determined as described previously (Putney *et al.* 1978).

The [^{3}H]QNB and ^{45}Ca were obtained from New England Nuclear Corp., Boston, Mass. Carbachol, methacholine (acetyl-β-methyl-choline), atropine, hyoscine and procaine were obtained from Sigma Chemical Co., St Louis, Mo. Substance P was obtained from Peninsula Laboratories, San Carlos, California, U.S.A.

RESULTS

In agreement with reports by others, binding of [^{3}H]QNB was slow, requiring about 30 min to ensure equilibrium (Yamamura & Snyder, 1974). When non-specific binding was subtracted, equilibrium binding of [^{3}H]QNB was saturable. In initial experiments, atropine (10^{-5} M) was used to inhibit specific binding and to measure non-specific binding. Scatchard analysis of data obtained in this manner

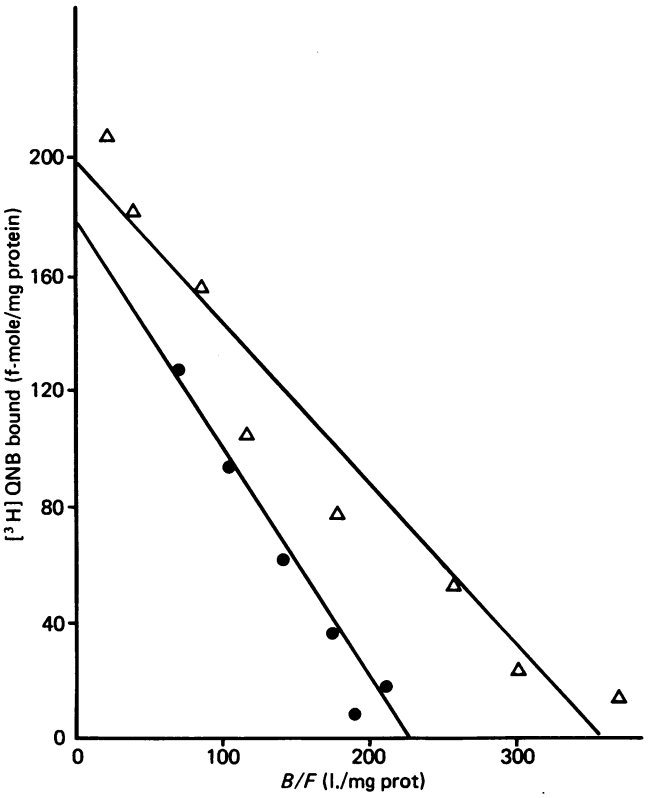

Fig. 1. Scatchard analysis of [^{3}H]QNB binding to dispersed rat parotid acinar cells. The quantity of [^{3}H]QNB bound was determined by incubating cells in medium containing 0·05–10 nM-[^{3}H]QNB. Non-specific binding was subtracted from the total binding and the differences taken as specific binding for graphical analysis. Non-specific binding was determined by measuring binding in the presence of an excess of unlabelled drug. The drugs used were: △, atropine (10^{-5} M); ●, methacholine (10^{-3} M). Each curve summarizes three experiments which were averaged and subjected to least-squares analysis from which the theoretical lines were derived. The correlation coefficients (r) were: atropine, 0·967; methacholine, 0·994.

is shown in Fig. 1 by the open triangles. The estimated number of sites was 197·5 f-mole/mg protein and the apparent dissociation constant was $5{\cdot}6 \times 10^{-10}$ M. Based on later observations (discussed below), these experiments were repeated with methacholine (10^{-3} M) used to measure non-specific binding. With these data, shown in Fig. 1 by filled circles, the number of binding sites was estimated to be 175·8 f-mole/mg protein, and the dissociation constant to be $7{\cdot}9 \times 10^{-10}$ M. The

latter figure agrees reasonably with previously reported binding values (Yamamura & Snyder, 1974) and pharmacological data (Birdsall & Hulme, 1976). For both procedures, the Hill plots had slopes near unity (atropine, 1·001; methacholine, 0·925; Fig. 2).

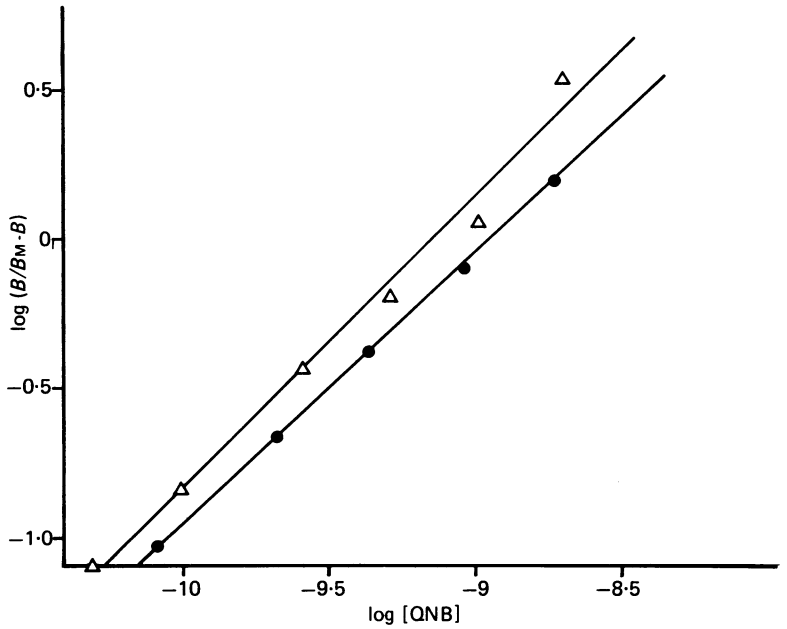

Fig. 2. Hill plot of the data from Fig. 1. The symbols have the same significance as for Fig. 1. The lines were fit by a least-squares analysis, and have the following correlation coefficients (r): atropine, 0·993; methacholine, 0·999.

The effects of varying concentrations of atropine, hyoscine, methacholine or carbachol on total [^{3}H]QNB binding are shown in Fig. 3. Each drug maximally inhibited 60–70% of the total binding, carbachol being a notable exception. In other experiments (not shown) substance P in a concentration more than sufficient to produce a maximal response (10^{-7} M) had no effect on [^{3}H]QNB binding. Similarly, Co (3 mM), an agent that blocks only the Ca-dependent phase of the K release response to carbachol (Marier, Putney & Van de Walle, 1978), did not affect [^{3}H]QNB binding. Procaine (1 mM), on the other hand, an agent that blocks both phases of the K release response, almost completely (95%) blocked specific [^{3}H]QNB binding.

Returning to the data in Fig. 3, it is difficult to explain the deviations from theoretical behaviour observed with atropine and carbachol. In the case of carbachol, for which the data seemed most aberrant, it is worth noting that the concentrations that blocked greater than 60% of binding were extremely high (3 and 10 mM). One interpretation of these deviations is that atropine slightly, and carbachol substantially inhibit nonspecific (or non-receptor) binding. This may explain the slight curvature in Fig. 1 when atropine was used and was the rationale for repeating the [^{3}H]QNB concentration curve with methacholine to suppress specific binding. Acceptance of this assumption leads to a decided improvement of the consistency of the data with the theoretical relationship (Fig. 1) and previous literature in this

area (see below). With these data, the concentration producing half-maximal inhibition of binding (EC50) was determined by Scatchard analysis for atropine, hyoscine and methacholine, and by graphic estimation for carbachol, assuming that maximum inhibition of specific binding was the same as for methacholine. Apparent K_D values (dissociation constant for the drug–receptor complex) were

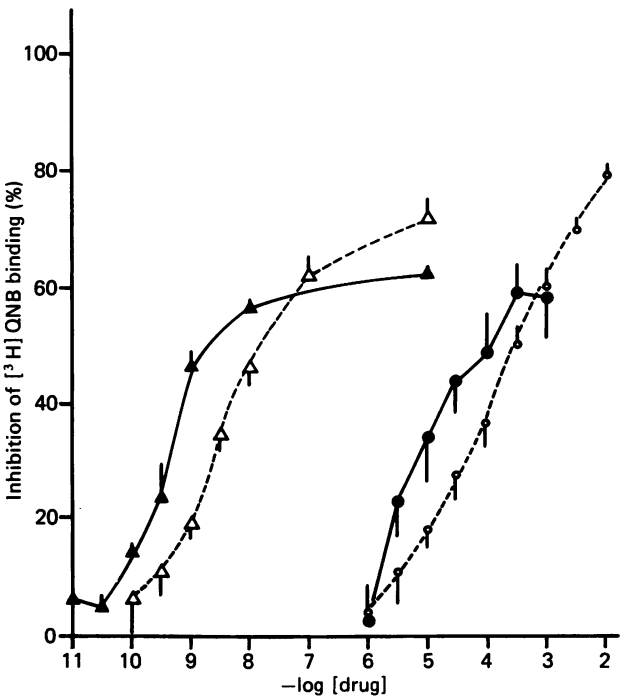

Fig. 3. Inhibition of [^{3}H]QNB binding by agonists and antagonists. Here, the % inhibition of total binding is given. The concentration of [^{3}H]QNB was 0·5 nM. The drugs used were: ▲, hyoscine (n = 3); △, atropine (n = 3); ●, methacholine (n = 3); ○, carbachol (n = 6). Dispersions represent one S.E. of mean.

TABLE 1. Apparent K_D (binding and K release) values for muscarinic agonists and antagonists in rat parotid gland cells. All values (with exception of the R ratio) are mole/l.

Drugs	K_D (binding)	K_D (K release)	R*	K_D (literature)	Ref.†
Hyoscine	$1{\cdot}96 \times 10^{-10}$	$1{\cdot}6 \times 10^{-10}$	1·22	$2–9 \times 10^{-10}$	A
Atropine	$8{\cdot}28 \times 10^{-10}$	$5{\cdot}2 \times 10^{-10}$	1·59	5×10^{-10}	B
		$(1{\cdot}0 \times 10^{-9})$‡		$1–4 \times 10^{-9}$	A
Carbachol	$1{\cdot}87 \times 10^{-5}$	$1{\cdot}0 \times 10^{-6}$	18·7	$2–3 \times 10^{-5}$	C
		$(1{\cdot}3 \times 10^{-6})$‡		$1{\cdot}5 \times 10^{-5}$	D
				2×10^{-5}	E
Methacholine	$2{\cdot}76 \times 10^{-6}$	$2{\cdot}2 \times 10^{-7}$	12·5	$2–5 \times 10^{-6}$	A
				1×10^{-5}	E

* $R = K_D$ (binding)/K_D (K release).

† References are: A, Snyder, Chang, Kuhar & Yamamura (1975); B, Paton & Rang (1965); C, Yamamura & Snyder (1974); D, Birdsall & Hulme (1976); E, Ward & Young (1977).

‡ Values from ^{86}Rb experiments with slices.

calculated from the relationship $K_D = EC50/(1+[QNB]/K_{QNB})$ which assumes that the inhibition by drugs of [^{3}H]QNB binding is competitive in nature (Gaddum, 1957; Schild, 1957). The resulting values are given in Table 1.

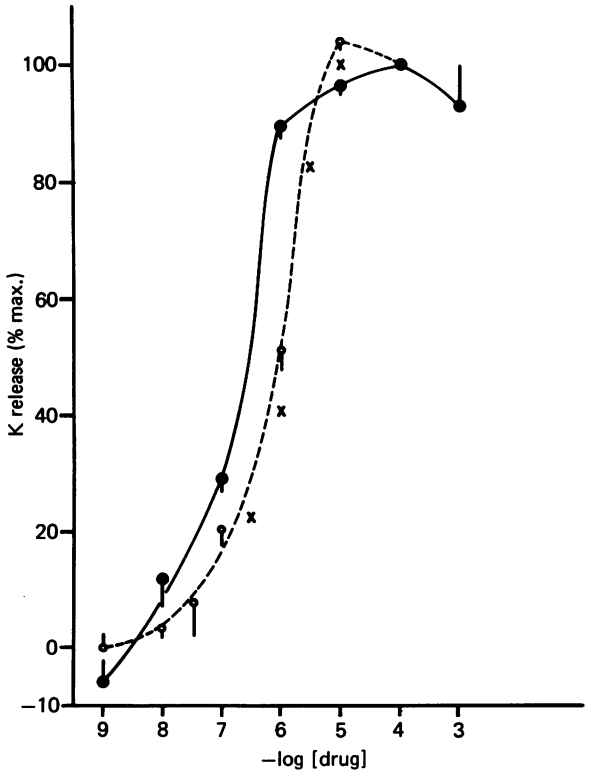

Fig. 4. Concentration–effect relationships for K release due to carbachol (○) or methacholine (●). Data are expressed as % of release obtained with 10^{-4} M drug and summarize four experiments for each agonist. Data obtained from ^{86}Rb experiments with slices are also included (×). Dispersions represent one S.E. of mean.

The cell preparation was used to determine the concentration–effect relationships for these same drugs in producing K release. The relationship for carbachol was determined with and without 10^{-8} M-atropine present, and that for methacholine, with and without 10^{-9} M-hyoscine present. From these data, K_D (EC50) values for the agonists were determined, and the K_D for the antagonists determined from the shift in apparent EC50 of the agonist curves (Arunlakshana & Schild, 1959). These values are given in Table 1. The concentration–effect data for carbachol and methacholine are shown in Fig. 4. The experiments with carbachol and atropine were repeated with slices and the ^{86}Rb release technique described previously (Putney, 1976). The apparent K_D values for carbachol and atropine from the slice preparation are also included in Table 1 and the carbachol data are included in Fig. 4. The agreement of values obtained with cells and slices suggests that receptor number and quality were not affected by the isolation procedure.

The agreement (or disparity) between K_D values for K release and K_D values for receptor binding was determined by calculation of the ratio $R = K_D$ (binding)/

K_D(K release), and these values are included in Table 1. There was reasonably good agreement between binding and pharmacological effect for antagonists, as indicated by R values close to unity. For agonists, however, ratios considerably greater than one were obtained.

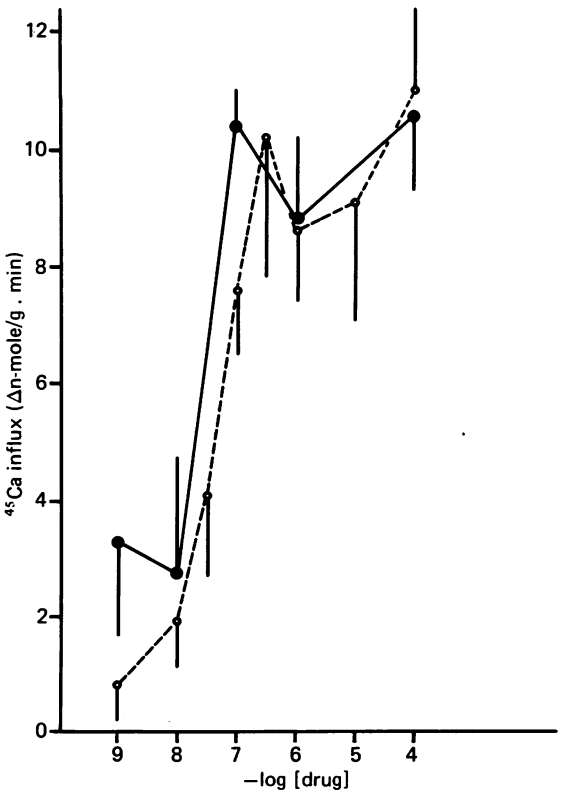

Fig. 5. Concentration–effect relationships for ^{45}Ca influx due to carbachol (○) or methacholine (●). Basal influx (*ca.* 40 n-mole/g.min) has been subtracted. Each curve summarizes ten experiments. Dispersions represent one S.E. of mean.

A primary concern of this study was the relationship between the muscarinic receptor and the Ca channel. K movements which can be measured with reasonable precision and statistical reliability may reflect changes in intracellular Ca following channel activation. However, when the binding and effect curves disagree, it is important to know whether channel activation correlates with binding or response data. Ideally, Ca influx would be a more direct measure of channel activation than K release. The maximum stimulation of Ca influx in parotid cells unfortunately represents a mere 15–20% increase over basal values, making quantitative concentration–effect determinations difficult. This is evident in the variability shown in Fig. 5 where concentration–effect data for ^{45}Ca influx due to carbachol and methacholine are summarized. Despite this variability, the concentration range of the ^{45}Ca influx response appears to lie between 10^{-8} and 10^{-6} M and thus seems better correlated with the K release data than with the ligand binding data.

DISCUSSION

The radiolabelled antagonist, [^{3}H]QNB, has been useful in studies of muscarinic receptors in a number of tissues (Yamamura & Snyder, 1974; Snyder *et al.* 1975; Birdsall & Hulme, 1976). Previous studies have usually employed membrane fractions for binding studies. The data presented here suggest that biochemical information on receptor binding in the rat parotid gland can also be obtained with living cells. Strittmatter, Davis & Lefkowitz (1977*a*, *b*) used a similar method in a study of α-adrenoceptors in the parotid gland. A problem encountered by these investigators was a failure of [^{3}H]dihydroergocryptine binding to saturate, despite good agreement on binding at lower concentrations between membranes and cells. A similar problem was encountered with carbachol inhibition of [^{3}H]QNB binding in this study. The reason for this anomalous behaviour is unclear, but the high concentration of carbachol required suggests that the effect is probably not receptor related.

On balance, however, the data indicate that [^{3}H]QNB gives a reasonable picture of muscarinic receptor binding in the parotid acinar cell. The evidence for this is: (1) linearity of Scatchard and Hill plots when methacholine is used to measure non-specific binding, (2) similarity between the experimentally measured dissociation constant for [^{3}H]QNB in parotid cells and literature values for binding to membrane receptors or inhibition of smooth muscle contraction (Yamamura & Snyder, 1974; Birdsall & Hulme, 1976), (3) close agreement between binding and pharmacological dissociation constants for atropine and hyoscine (Table 1), and (4) reasonable agreement between the experimentally determined binding constants for all of the compounds and those from previous studies with membranes (Table 1).

The discrepancy between receptor binding of agonists and biological effect has also been observed in other studies (Birdsall & Hulme, 1976; Birdsall, Burgen, Hiley & Hulme, 1976; Furchgott, 1978; Ward & Young, 1977). In addition, muscarinic agonists (but not antagonists) in smooth muscle and brain routinely give Hill coefficients considerably less than one. Whether this indicates separate populations of receptors or a complex mechanism of agonist binding has not yet been resolved (Birdsall *et al.* 1976; Ward & Young, 1977). In this study, Hill plots of methacholine and scopolamine binding gave values of 1·04 and 0·98 respectively.

If the receptor population in the parotid is homogeneous, then one explanation for the discrepancy between binding and effect curves for agonists is the phenomenon of spare receptors; i.e. only a fraction of total receptors need be activated to produce a maximal response (Stephenson, 1976). Furchgott (1978) has discussed how irreversible inactivation of a fraction of receptors can yield a pharmacological estimate of 'true' receptor binding. His data suggest that carbachol binding to the muscarinic receptor should have a dissociation constant of $1\text{–}2 \times 10^{-5}$ M, in good agreement with binding data of others and of this study (Table 1).

The term 'spare receptors' is a purely descriptive one and says nothing about the nature of the quantitatively limiting entity (or entities). The β-adrenoceptor mechanism in the parotid gland shows a similar phenomenon (Butcher, Goldman & Nemerovski, 1975; Au, Malbon & Butcher, 1977). In this instance, however, receptor binding and adenyl cyclase activation are fairly well correlated, while amylase secretion occurs in a lower concentration range. One explanation for these data is

that in the case of the β-adrenoceptor mechanism, the spare receptor phenomenon reflects the fact that small increases in cyclic AMP can mediate maximal secretory responses.

A similar mechanism could occur for the muscarinic receptor although dose–effect data for the appropriate second messenger, Ca influx, are not so easily obtained. With the dispersed cell preparation, ^{45}Ca influx can be directly measured (Putney *et al.* 1978), although small net increases make for high variability in the data. Still, the data shown in Fig. 5 show that Ca influx is stimulated in a concentration range (10^{-8}–10^{-6} M) far below that for ligand binding (10^{-6}–10^{-4} M). This evidence suggests that spare muscarinic receptors may occur without 'spare' Ca channels; i.e. the availability of Ca channels may be rate-limiting. In support of this, it has previously been shown that 10^{-5} M-carbachol produces a maximum response, specifically for the Ca-dependent phase of the K release response, in media containing 1·0 mM-Ca (Putney, 1976). A greater response can be obtained by increasing extracellular Ca to 3·0 mM (Marier *et al.* 1978). Since these data show that Ca availability is rate-limiting when Ca is 1·0 mM, it seems reasonable to assume that 10^{-5} M-carbachol must activate all of the available Ca channels.

A quantitative consideration of receptor density is of interest, especially in light of previously proposed theories of the relationship between receptors and Ca channels (Putney, 1977; Marier *et al.* 1978). The average protein content per cell was estimated to be 0·214 ng. Accordingly, the average receptor density would be 23,000 sites/cell which is similar to the previously reported density of α-adrenoceptors of 15,000 sites/cell (Strittmatter *et al.* 1977*a*, *b*). In the case of methacholine, however, the agonist for which the most reliable data are available, the displacement in binding and effect curves would suggest that as few as 8% (1/12·5) of these receptors, or 1800/cell can produce a full response. Even conceding a possible twofold error in this estimate, this value differs markedly from the reported number of α-adrenoceptors. For the α-adrenoceptors, binding and effect curves coincided, suggesting that all 15,000 sites were necessary to activate a full response.

This disparity in site numbers between muscarinic receptors and α-adrenoceptors is interesting, especially in light of the evidence that these two receptors activate the same population of Ca channels (Putney, 1977; Marier *et al.* 1978). Whether this indicates a qualitative or quantitative difference in the mechanisms of receptor–channel interactions cannot be determined. Another receptor, for substance P, controls these same channels and it would be useful to have quantitative information on these sites as well.

A previous theory on the action of procaine was examined by ligand binding measurements. In a previous report, procaine was shown to block muscarinic and α-adrenergic responses, but not substance P responses. It was suggested that the local anaesthetic interferes with some step between receptor occupation and channel activation (Marier *et al.* 1978). Receptor inhibition was considered unlikely, since muscarinic and α-adrenergic mechanisms were similarly affected. The ligand binding experiments reported here suggest that such conjecture, though reasonable, may be incorrect. Procaine, at a concentration similar to that employed previously, almost completely blocked [^{3}H]QNB binding, suggesting that for the muscarinic mechanism, receptor blockade can explain the inhibitory actions of the local anaesthetic. As

expected, the divalent cation, Co, which specifically blocks only the Ca-dependent phase of the K permeability response regardless of the agonist employed has no apparent effect on muscarinic receptor binding. This is consistent with the idea that Co acts specifically at the Ca channel level.

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