A POSSIBLE ROLE OF SYNAPTIC-MEMBRANE PROTEIN PHOSPHORYLATION IN THE REGULATION OF MUSCARINIC ACETYLCHOLINE RECEPTORS

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1. Introduction

A decrease in the number of receptors detectable by the use of ligand-binding assays has often been shown to occur as a consequence of prolonged exposure of receptors to their particular agonists [1]. This phenomenon which has been termed receptor 'subsensitivity' or 'desensitisation' has been particularly well studied in the case of the β-adrenergic receptor [2–7]. A loss of β-adrenergic receptors has been shown following exposure of erythrocytes [2,3] and cultured mouse lymphoma cells [4] to β-adrenergic agonists, as a result of circadian changes in noradrenaline release in the pineal gland [5,6], and as a consequence of drug treatment in experimental animals [7]. While the mechanism underlying this receptor loss is not fully understood, recent work has suggested a role for receptor internalisation [8]. Similar receptor loss or densensitisation has been reported for α-adrenergic [9], nicotinic cholinergic [10] and muscarinic cholinergic receptors [11,12] as well as several polypeptide hormone receptors [1] following prolonged exposure to agonists. A reduction in muscarinic receptor number has been demonstrated to occur following depolarisation of a synaptosomal preparation [13]. Since synaptosomal depolarisation is known to result in increased phosphorylation of particular synaptic-membrane proteins [14,15] it was decided to examine membrane phosphorylation as a possible mechanism for receptor desensitisation. Evidence is presented here which indicates that membrane protein phosphorylation has no effect on β-adrenergic receptors. However, a time-dependent, cyclic AMP (cAMP)-stimulated reduction in the number of muscarinic receptors apparently due to synaptic membrane protein phosphorylation was found.

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2. Materials and methods

Synaptic membranes from 50 day old male Wistar rats were prepared as in [18,19]. For phosphorylation [20,21] membranes were suspended in 50 mM Tris–HCl (pH 8.0), 1 mM $MgCl_2$ to 0.2–0.4 protein mg/ml and incubated at 37°C for 5 min with no additions 1 mM ATP or 1 mM ATP + 50 μM cAMP. The reaction was terminated by addition of ice-cold buffer, the membranes rapidly pelleted by centrifugation at 45 000 × *g* for 5 min, washed and resuspended in the appropriate buffer for the binding assay. For assay of the muscarinic receptor [16] 20–40 μg membrane protein was incubated in 2 ml/50 mM sodium phosphate (pH 7.4) containing 5 nM [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB, spec. act. 16 Ci/mmol; Radiochemical Centre, Amersham). Three samples were incubated with and 3 without 1.25 μM atropine sulphate. Samples were incubated at 25°C for 30 min, the reaction terminated by addition of ice-cold buffer, the samples filtered under vacuum through 2.5 cm Whatman GF/B glassfibre filter discs and washed 3 times. The discs were counted in scintillation Cocktail T (Hopkin and Williams, Essex). Specific binding of [^{3}H]QNB was calculated as the mean amount bound in the absence of atropine sulphate minus that bound in its presence and expressed as pmol bound/mg protein.

For assay of the β-adrenergic receptor [17] 100–200 μg membrane protein was incubated in 1 ml 50 mM Tris–HCl (pH 8.0) containing 0.5 nM [^{3}H]-dihydroalprenolol ([^{3}H]DHA, spec. act. 42.5 Ci/mmol;

Radiochemical Centre, Amersham). Three samples were incubated with and 3 without 1 μM L-isoproterenol–D-bitartrate. Samples were incubated at 25°C for 30 min, the reaction terminated by addition of ice-cold buffer, and samples filtered and washed. Specific binding of [^{3}H]DHA was calculated as the mean amount bound in the absence of isoproterenol minus that bound in its presence and expressed as fmol bound/mg protein.

For the incorporation of phosphate from [γ-^{32}P]-ATP, synaptic membranes were incubated under identical conditions to those indicated above for protein phosphorylation except for the addition of 10 μCi [γ-^{32}P]ATP (spec. act. 2800 Ci/mmol; Radiochemical Centre, Amersham) per 100 μl reaction mixture. Incubations were at 37°C for varying times with or without cAMP, the reaction stopped by the addition of 100 μl dissociation buffer (125 mM Tris–HCl (pH 6.8), 1.25% SDS, 1% 2-mercaptoethanol, 10% sucrose, 2 mM EDTA), proteins solubilised by heating to 100°C for 1 min and 100 μl aliquots analysed by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis on 8% slab gels. Autoradiography was carried out on dried gels. In some cases reactions were stopped by the addition of 10% Trichloroacetic acid for the determination of total radioactivity bound to trichloroacetic acid-precipitable material [20].

3. Results

3.1. *Effect of phosphorylation on ligand binding*

The effect of synaptic-membrane phosphorylation on muscarinic and β-adrenergic receptors was determined by examining the specific binding of the muscarinic antagonist, [^{3}H]QNB, and the β-adrenergic antagonist, [^{3}H]DHA, to synaptic-membranes preincubated for 5 min in the presence of 1 mM ATP or 1 mM ATP + 50 μM cAMP in conditions optimal for protein phosphorylation. The data shown in fig.1 indicates that preincubation under these conditions has no effect on [^{3}H]DHA binding. However the binding of [^{3}H]QNB is reduced by ~20% by preincubation in the presence of ATP and reduced even further (by ~40%) when cAMP is also present during the preincubation; cAMP alone had no effect on the level of [^{3}H]QNB binding. The [^{3}H]QNB binding assay was carried out at a saturating concentration of [^{3}H]QNB and therefore a reduction in binding is likely to be due to a decrease in receptor number rather than binding affinity. That this is the case was confirmed by Scatchard analysis of [^{3}H]QNB binding to control membranes and membranes preincubated with ATP (fig.2). The K_d-values calculated from the Scatchard plots were virtually identical (control K_d = 0.328 nM; +ATP K_d = 0.330 nM) while the B_{max} value was reduced by preincubation with ATP (control, B_{max} = 1.52 pmol/mg protein; +ATP, B_{max} = 1.34 pmol/mg protein).

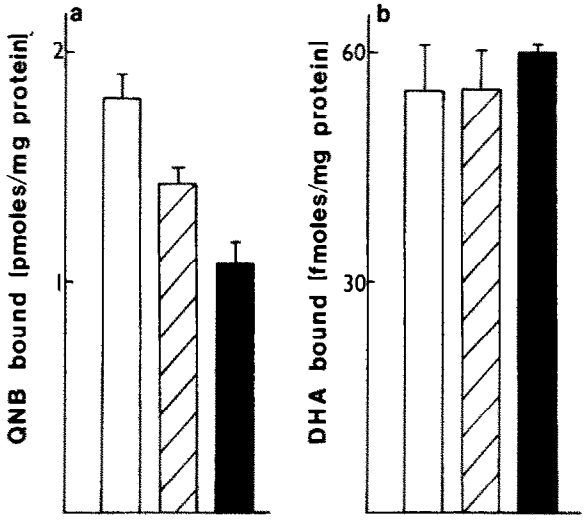

Fig.1. Effect of synaptic membrane phosphorylation on the binding of [^{3}H]QNB (a) and [^{3}H]DHA (b) to synaptic membranes of rat cerebral cortices. Membranes were preincubated at 37°C for 5 min with no additions (□), 1 mM ATP (▨) or 1 mM ATP + 50 μM cAMP (■). Data shown for [^{3}H]QNB binding as mean ± SEM for 4 independent expt. and for [^{3}H]-DHA for 5 expt. Statistics: for [^{3}H]QNB binding ATP *vs* control, p 0.02; ATP + cAMP *vs* control, $p < 0.002$; ATP *vs* ATP + cAMP, $p < 0.05$ (unpaired Student's t-test).

A reduction in [^{3}H]QNB binding could have been caused by a direct inhibitory effect of ATP not removed by the washing procedure carried out prior to the binding assay. This is unlikely to be the case since the decrease in [^{3}H]QNB binding was found to be time-dependent (fig.3). Furthermore, a 10-fold increase in the concentration of ATP used in the preincubation (to 10 mM) which would lead to an increase in the amount of any ATP left after washing did not result in any greater decrease in receptor binding. Incidentally, endogenous protein kinase activity was shown to be maximal at 1 mM ATP [20].

Examination of the nucleotide specificity of the decrease in number of muscarinic receptors was exam-

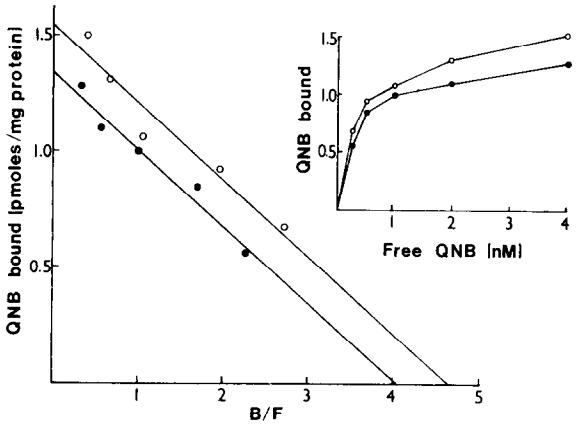

Fig.2. Scatchard plot and saturation curve (inset) of specific [^{3}H]QNB binding to synaptic membranes. Synaptic membranes were preincubated for 5 min with 1 mM ATP (•) or without additions (control (○)). Conditions for preincubation and [^{3}H]QNB binding were as indicated in legend to fig.1. B/F = {bound [^{3}H]QNB (pmol/mg protein)}/{free [^{3}H]QNB (nM)}.

ined by substitution of ATP or cAMP by GTP or cGMP, respectively. This experiment showed that neither GTP nor cGMP were effective (not shown).

The decrease in receptor number is probably due to membrane protein phosphorylation rather than any other ATP-dependent mechanism. This interpretation is supported by the stimulatory effect of cAMP on the phenomenon. The involvement of protein kinase activity is further indicated by the dependence of the phenomenon on the presence of Mg^{2+} during preincubation (protein kinase activity is magnesium-dependent [21]) and the abolition of receptor loss by preincubation in the presence of a concentration of Na^+ that has been shown to inhibit protein kinase activity [21] (table 1).

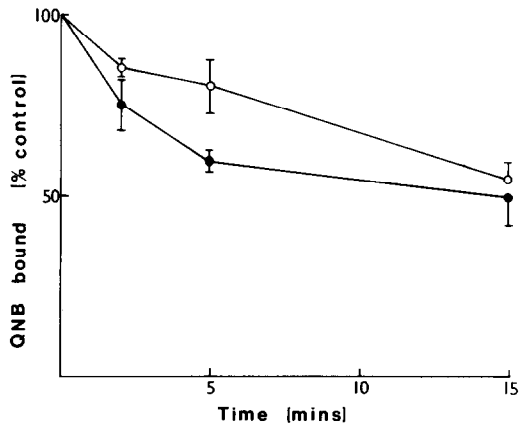

Fig.3. Time course of decrease in [^{3}H]QNB binding due to preincubation with 1 mM ATP (○) or 1 mM ATP + 50 μM cAMP (•). Synaptic membranes were preincubated with added ATP or ATP + cAMP or without additions (control) for varying times. Data was calculated as specific [^{3}H]QNB bound (pmol/mg protein) and are expressed as mean % of control value + SEM from 5 independent expt. Statistics: 5 min ATP *vs* ATP + cAMP, $p < 0.05$. Incubation at 37°C had little effect on the levels of [^{3}H]QNB binding in control membranes over the time indicated, <10% of binding being lost.

Table 1
Effect of Mg^{2+} and Na^+ on decrease in [^{3}H]QNB binding due to preincubation with ATP

Mg^{2+} present	Additions to preincubation reaction mixture	Specific [^{3}H]QNB binding	
		(pmol/mg protein)	(% control)
+	–	1.79	100
+	1 mM ATP	1.00	56.0
+	1 mM ATP + 100 mM NaCl	1.71	95.5
+	–	1.71	100
+	1 mM ATP	1.31 ± 0.01	80.4 ± 5.4
–	–	1.95	100
–	1 mM ATP	1.91	97.0

Synaptic membranes were preincubated for 15 min with or without 1 mM ATP in the presence or absence of 1 mM $MgCl_2$ or 100 mM NaCl as indicated. Conditions of preincubation and details of [^{3}H]QNB binding assay as in section 2. Specific [^{3}H]QNB binding was expressed as pmol bound/mg protein and as a percentage of control values. Data are means (or mean ± SEM) from 2 (or 3) determinations

It was attempted to confirm the above interpretation by using the specific protein inhibitor of cAMP-dependent protein kinase. However, this inhibitor was found to be without effect on endogenous phosphorylation in synaptic membranes. This lack of effect was probably due to inaccessibility of protein kinase to the inhibitor in intact synaptic membranes.

3.2. *Characteristics of membrane phosphorylation*

Examination of total bound ^{32}P to synaptic membranes after incubation with [γ-^{32}P]ATP (fig.4a) indicated a peak in the level of phosphorylation after incubation for 2.5–5.0 min and a subsequent 20% drop in the level of phosphorylation following continued incubation. It is noteworthy that incubation of phosphorylated synaptic membranes at 25°C for 30 min in the receptor assays did not result in any

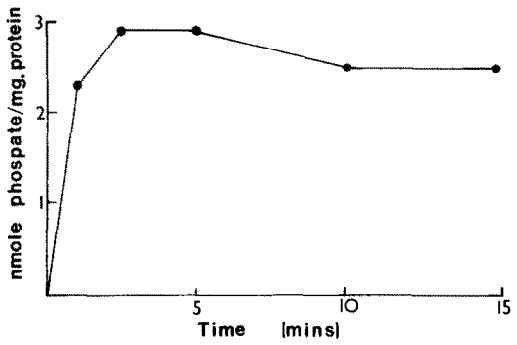

Fig.4. Time course of phosphorylation of synaptic membranes. Total ^{32}P incorporation into trichloroacetic acid-precipitable material following incubation with 10 μCi [γ-^{32}P]ATP, 1 mM ATP, 50 μM cAMP is shown.

detectable membrane dephosphorylation (not shown) as would be expected in the absence of Mg^{2+} [21]. Separation of membrane polypeptides by SDS–polyacrylamide gel electrophoresis indicated an overall stimulation of protein phosphorylation as well as a much greater stimulation of the phosphorylation of particular polypeptides by cAMP (not shown). The major phosphoproteins all showed a peak of phosphorylation at 2.5–5.0 min with a subsequent drop in the level of phosphorylation following further incubation. These results were similar to those in [20].

4. Discussion

The lack of effect of synaptic-membrane phosphorylation on [^3H]DHA binding is consistent with the finding that isoproterenol-induced receptor desensitisation still occurs in cells lacking cAMP-dependent protein kinase activity [4]. However, β-adrenergic receptor loss in erythrocytes has been associated with the increased phosphorylation of two membrane proteins [8]. In the case of the muscarinic receptor, prolonged exposure of the neuron-like hybrid cell line NG108-15 to the muscarinic agonist carbachol leads to an increase in cAMP levels as well as receptor loss [11]. Thus a cAMP-stimulated protein kinase activity could be the mechanism underlying loss of muscarinic receptors. Interestingly, some of those proteins whose phosphorylation is stimulated by cAMP are most probably identical to those whose phosphorylation is stimulated by depolarisation [15]; depolarisation itself results in a loss of muscarinic receptors [13].

The data presented here indicate that incubation of synaptic membranes under phosphorylating conditions leads to a cAMP-stimulated loss of muscarinic receptors. The Mg^{2+}-dependency and Na^+-sensitivity of this receptor loss argue for the involvement of protein kinase activity. However in comparing the time course of receptor loss (fig.3) and that of membrane protein phosphorylation (fig.4a,b) a disparity is evident in that while the major phosphoproteins all show to some degree a reduction in the level of phosphorylation following incubation at 37°C for >5 min, the muscarinic receptor loss was maintained following incubation for up to 15 min. One explanation for this apparent disparity in the time courses may be that receptor loss is brought about by the phosphorylation of a minor membrane component, such as the muscarinic receptor itself, with a longer-lasting time course of phosphorylation. Examination of the degree of phosphorylation of a membrane component as sparse as the muscarinic receptor (amounting to ~0.01% of total membrane protein [23] is obviously difficult with a membrane as complex as the synaptic-membrane. Alternatively phosphorylation-mediated receptor loss may not be brought about by phosphorylation of the receptor itself but by a multi-component process not immediately reversed by dephosphorylation.

While I would like to suggest a possible role for protein phosphorylation in the regulation of the muscarinic acetylcholine receptor, the definitive experiments may have to await the purification of the receptor and a direct examination of its phosphorylation state as has been done for the nicotinic acetylcholine receptor [24].

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