THE LOSS OF MUSCARINIC ACETYLCHOLINE RECEPTORS IN SYNAPTIC MEMBRANES UNDER PHOSPHORYLATING CONDITIONS IS DEPENDENT ON CALMODULIN

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1. Introduction

A reduction in the apparent number of muscarinic acetylcholine receptors has been shown to occur as a consequence of prolonged exposure to muscarinic agonists in cell culture [1–4], drug treatments in experimental animals [5], and depolarisation of synaptosomes [6]. It had been suggested that such 'down regulation' of the muscarinic receptor may be mediated by a protein phosphorylation mechanism [7]. This suggestion was based on the finding that preincubation of synaptic-membranes under phosphorylating conditions led to a loss in the apparent number of muscarinic binding sites measured in a subsequent ligand-binding assay. This receptor loss was time-dependent and stimulated by cyclic AMP (cAMP) [7].

Calmodulin is a small heat-stable calcium-binding protein demonstrated to be involved in a wide range of systems regulated by Ca^{2+} [8,9]. Immunocytochemical techniques have demonstrated its presence at central synapses [10,11]. Calmodulin remains firmly bound to synaptic membranes but can be removed by treatment with Ca^{2+}-chelating agents [12,13]. Using such calmodulin-depleted membranes a calmodulin-dependent, calcium-activated protein phosphorylation system has been found in synaptic membranes from rat cerebral cortex [14].

The results of this paper indicate that the loss of muscarinic receptors as a consequence of preincubation under phosphorylating conditions is dependent on the presence of calmodulin.

2. Materials and methods

Synaptic membranes from rat cerebral cortices were prepared as in [15,16] except that all sucrose solutions were buffered in 5 mM Tris–HCl (pH 8.4) with either 50 μM $CaCl_2$ or 1 mM EDTA. After fractionation the membranes were washed once in 5 mM Tris–HCl (pH 8.4) + either 50 μM $CaCl_2$ or 1 mM EDTA. For the preincubation under phosphorylating conditions, membranes were resuspended in 50 mM Tris–HCl (pH 8.0), 1 mM $MgCl_2$, 50 μM $CaCl_2$ (0.4 0.6 mg protein/ml) and incubated at 37°C for 5 min in the presence or absence (control) of 1 mM ATP, 50 μM cAMP. In experiments in which the effect of exogenous calmodulin or protein kinase was examined the membranes were preincubated with the added protein at 4°C for 30 min before addition of the ATP/cAMP. Following incubation the membranes were pelleted at 14 000 × *g* for 3 min in an Eppendorf microfuge, washed and resuspended in 50 mM sodium phosphate (pH 7.4). Specific binding of L-[^{3}H]quinuclidinyl benzilate ([^{3}H]QNB, spec. act. 43 Ci/mmol; Radiochemical Centre, Amersham) was assayed as in [7] at a final concentration of 1 nM.

Two preparations of calmodulin were used in these experiments. Bovine brain calmodulin purified to homogeneity as in [17] was a gift from Dr C. Michison (Division of Biochemistry, NIMR). Rat brain calmodulin was prepared by essentially the method devised for the purification of calmodulin from phosphorylase kinase [18]. Rat cerebral cortices were homogenised in 5 mM Tris–HCl (pH 8.4), 1 mM EDTA and centrifuged at 45 000 × *g* for 30 min. The supernatant was made up to 15 mM mercaptoethanol heated in a boiling water bath for 5 min, rapidly cooled and centrifuged at 45 000 × *g* for 30 min. The supernatant was reboiled, recentrifuged and fractionated [18] on a 7 × 1.5 cm column of DEAE–Sephadex A-50 (Pharmacia). Preparations made in this way were

found by SDS–polyacrylamide gel electrophoresis to contain 60–85% calmodulin. Trifluoperazine was a gift from Smith, Kline and French Labs. Cyclic AMP-dependent protein kinase (predominantly type II from bovine heart) was purchased from Sigma.

Incorporation of phosphate from [γ-^{32}P]ATP into synaptic membranes, determination of total incorporation into trichloroacetic acid-precipitable material, SDS–polyacrylamide gel electrophoresis of samples on 8% slab gels and autoradiography were done as in [7].

Calcium buffers containing 5 mM EDTA, 5 mM $MgCl_2$ and varying [$CaCl_2$] were prepared from calculations based on the dissociation constants of EDTA–Ca^{2+} and EDTA–Mg^{2+} using a computer program devised by Dr M. Geisow (Division of Biophysics, NIMR) and final free [Ca^{2+}] determined by comparison to standard solutions of Ca^{2+} (BDH) using a calcium electrode [19]. The values for free Ca^{2+} given in the text are those derived empirically.

3. Results

3.1. *Effect of preincubation under phosphorylating conditions on muscarinic receptors in membranes prepared in the presence of Ca^{2+} or EDTA*

It had been shown that preincubation under phosphorylating conditions led to a loss in the level of binding of the muscarinic antagonist [^{3}H]QNB to synaptic membranes prepared in the absence of exogenous Ca^{2+}. A similar loss in [^{3}H]QNB-binding was found here following preincubation of membranes prepared in the presence of 50 μM Ca^{2+} (table 1). However, no loss in [^{3}H]QNB was found following preincubation with ATP + cAMP of synaptic membranes prepared in the presence of 1 mM EDTA. This difference between membranes prepared under the two conditions was not due to differences in the level of Ca^{2+} itself since all the preincubations in table 1 were done at 50 μM Ca^{2+}. Abolition of the phenomenon of receptor loss could also be brought about by washing membranes prepared in the presence of Ca^{2+} with either 1 mM EDTA or 1 mM EGTA (not shown).

3.2. *Effect of exogenous calmodulin on phosphorylation-induced receptor loss in membranes prepared in the presence of EDTA*

The abolition of phosphorylation-induced receptor loss by preparation of membranes in the presence of EDTA was not due to a direct effect on cAMP-dependent protein phosphorylation. This is shown by the fact that the basal level of phosphate incorporation from [γ-^{32}P]ATP and the magnitude of the cAMP-stimulation of this incorporation were no different in membranes prepared in the presence of Ca^{2+} or EDTA (not shown). Furthermore recovery of phosphorylation-induced receptor loss was not brought about by addition of exogenous cyclic AMP-dependent protein

Table 1
Effect of presence of Ca^{2+} and EDTA during membrane preparation and presence of exogenous protein kinase or calmodulin on muscarinic receptor loss

Additions during membrane preparation	Additions during preincubation	[^{3}H]QNB binding (% of control)
50 μM Ca^{2+}	–	86.5 ± 1.38[a] (13)
1 mM EDTA	–	100.1 ± 1.37 (11)
1 mM EDTA	10 μg protein kinase/ml	99.3 ± 1.66 (3)
1 mM EDTA	15 μg calmodulin/ml	84.1 ± 1.88[b] (7)

[a] $p < 0.001$, [b] $p < 0.01$, unpaired Student's t-test

Synaptic membranes were prepared in the presence of either 50 μM Ca^{2+} or 1 mM EDTA and preincubated in the presence or absence (control) of 1 mM ATP, 50 μM cAMP. In some cases cyclic AMP-dependent protein kinase or calmodulin were added to the preincubation. The specific binding of [^{3}H]QNB was determined and expressed as a percentage of control. Data is shown as mean ± SEM of the number of determinations shown in parentheses

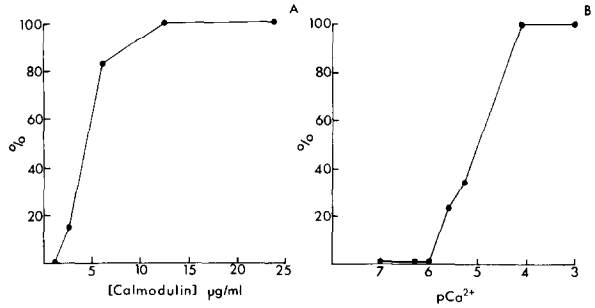

Fig.1. Dependence of phosphorylation induced muscarinic receptor loss on (A) calmodulin at 100 μM Ca^{2+} and (B) Ca^{2+} at 24 μg calmodulin/ml. The binding of [^{3}H]QNB was determined following preincubation in the presence of 1 mM ATP, 50 μM cAMP and varying of free [Ca^{2+}] (using an EDTA/Mg^{2+}/Ca^{2+} buffer) and calmodulin at 37°C for 5 min. The loss in [^{3}H]QNB binding at each [Ca^{2+}] or calmodulin was expressed as a percentage of the maximum receptor loss.

kinase to EDTA-prepared membranes (table 1). However, a recovery of phosphorylation-induced receptor loss was found following addition of either rat brain or bovine brain calmodulin to the preincubation (table 1). The loss in [^{3}H]QNB binding was dependent on calmodulin concentration and was calcium-dependent (fig.1).

Calmodulin itself had no direct effect on [^{3}H]-QNB-binding since membranes prepared in the presence of Ca^{2+} or EDTA gave mean levels of binding/mg protein in 5 preparations within 1% of each other. Furthermore preincubation with calmodulin alone without the addition of ATP had no effect on the level of [^{3}H]QNB binding.

Trifluoperazine has been shown to bind to calmodulin with high affinity [20] and has been used as a specific calmodulin antagonist [8]. I was unable to show a specific effect of trifluoperazine on phosphorylation-induced receptor loss since preincubation with 100 μM trifluoperazine, a level shown to inhibit calmodulin-stimulated protein kinase activity [21,22], completely abolished [^{3}H]QNB binding. This effect may be related to the hydrophobicity of trifluoperazine [8] and a direct interaction with the membranes.

3.3. *Effect of calmodulin on membrane protein phosphorylation*

In an attempt to clarify the mechanisms underlying the calmodulin dependence of phosphorylation-induced receptor loss, the effects of calmodulin on phosphate incorporation from [γ-^{32}P]ATP into synaptic membranes was examined. Calmodulin stimulated phosphorylation in a calcium-dependent manner. The levels of calmodulin and Ca^{2+} for half-maximal stimulation of protein phosphorylation were determined and found to be similar to those for phosphorylation-induced receptor loss (table 2). It had been shown that muscarinic receptor loss under phosphorylating conditions was stimulated by cAMP [7]. Since the receptor loss now appears to be also calmodulin-dependent, the effect of calmodulin on cyclic AMP-dependent phosphorylation was examined in order to see if any synergism existed between the calmodulin- and cAMP-dependent systems. From the levels of phosphate incorporation into total trichloroacetic acid-precipitable material in the presence of cAMP, calmodulin or cAMP + calmodulin (table 3) it is evident that the stimulatory effects of cAMP and calmodulin are simply additive. This conclusion was borne out by examination of individual phosphoproteins by SDS–polyacrylamide gel electrophoresis (not shown). A lack of interaction between cAMP- and calmodulin-stimulated phosphorylation systems has been reported [23].

Table 2
Concentration of calmodulin and Ca^{2+} required for half-maximal stimulation of protein phosphorylation and loss in [^{3}H]QNB binding under phosphorylating conditions

μM	[^{3}H]QNB binding loss	Phosphorylation
Calmodulin	0.27	0.25
Ca^{2+}	12	10

Binding of [^{3}H]QNB and ^{32}P incorporation into total membrane protein were determined following incubation of synaptic membranes at 37°C for 5 min in the presence of 1 mM ATP, 50 μM cAMP and a range of [calmodulin] at saturating free [Ca^{2+}] (100 μM) or a range of free [Ca^{2+}] at a saturating [calmodulin] (1.4 μM). The [calmodulin] and [Ca^{2+}] giving 50% of the maximal stimulation are shown. The [calmodulin] was calculated on the basis of M_r 16 700

4. Discussion

These findings arose from the observation that phosphorylation-induced loss of [^{3}H]QNB binding to muscarinic receptors could not be detected in synaptic membranes treated with Ca^{2+}-chelating agents. Calmodulin is a known component of synaptic mem-

Table 3
Stimulation of phosphorylation of synaptic membranes by cAMP, calmodulin and calmodulin + cAMP

Additions	% of control	
	Predicted value	Actual value
None	–	100
cAMP	–	161.9
Calmodulin	–	125.6
Calmodulin + cAMP	187.5	191.0

The level of incorporation of ^{32}P from [γ-^{32}P]ATP into trichloroacetic acid-precipitable material following incubation at 37°C for 5 min in the presence of 1 mM ATP or with added cAMP (50 μM), calmodulin (24 μg/ml) or calmodulin + cAMP was determined. The levels of incorporation are expressed as a percentage of the incubation with no additions. The predicted value is that expected if the stimulation due to cAMP + calmodulin is a sum of that due to cAMP and that due to calmodulin alone

branes [10,11] and can be removed by EDTA or EGTA treatment [12–14]. Phosphorylation-induced receptor loss was found in EDTA-treated membranes incubated in the presence of exogenous calmodulin and Ca^{2+} indicating an obligate requirement for calmodulin in phosphorylation-induced receptor loss. It is noteworthy that sufficient calmodulin must remain associated with synaptic membranes prepared without the addition of exogenous Ca^{2+} since phosphorylation brought about a reduction in muscarinic receptors in synaptic membranes prepared in its absence [7].

In [14] 0.3 μM Ca^{2+} was required for half-maximal stimulation by calmodulin of phosphorylation in synaptic membranes, a value which is inconsistent with the known affinity constants for the binding of Ca^{2+} to calmodulin (4 μM for the high and 12 μM for the low affinity sites [24]). The value found here (10 μM) is consistent with a requirement of occupation by Ca^{2+} of both the high and low affinity sites on calmodulin for activation of protein kinase. Similarly the concentrations of calmodulin determined here to give half-maximal stimulation of receptor loss and protein phosphorylation are consistent with the known binding constants for calmodulin binding to striatal membranes (0.13 μM and 0.29 μM for high and low affinity sites, respectively [13]).

The levels of Ca^{2+} and calmodulin required for half-maximal stimulation of receptor loss and protein phosphorylation were found to be almost identical. This suggests that the calmodulin involvement in muscarinic receptor loss is at the level of calmodulin-dependent phosphorylation. However, the similarities in concentration dependence of receptor loss and phosphorylation could be coincidental.

It had been shown that muscarinic receptor loss following preincubation under phosphorylating conditions was stimulated by cAMP [7] and now phosphorylation-induced receptor loss has been found to be calmodulin-dependent. These findings could be accounted for by the following mechanisms for receptor inactivation:

A. Phosphorylation of a substrate protein (muscarinic receptor or an associated protein) by a single protein kinase stimulated by both calmodulin and cAMP.
B. Phosphorylation in a sequential fashion involving cAMP-dependent protein kinase, calmodulin-dependent protein kinase and substrate protein.
C. Phosphorylation of a substrate protein independently by both cAMP-dependent and calmodulin-dependent protein kinases.
D. Phosphorylation by a cAMP-dependent protein kinase and direct interaction of calmodulin with the substrate protein itself for inactivation.

Since the muscarinic receptor is a minor membrane component and its phosphorylation cannot be investigated directly, attempts to distinguish between the above mechanisms must be based on information regarding the general pattern of synaptic membrane phosphorylation. From such considerations we can eliminate mechanisms A and B since each predicts a synergistic interaction between the cAMP- and calmodulin-dependent phosphorylation systems. The data of table 2 indicates that in general such an interaction does not occur (see also [23]).

A precedent exists for mechanism C in the form of the synaptic phosphoprotein I which has been shown to be phosphorylated by both cAMP- and Ca^{2+}-dependent mechanisms [25]. The closeness of the levels of calmodulin and Ca^{2+} required for half-maximal stimulation of both muscarinic receptor loss and protein phosphorylation can be used as an argument for the involvement of a calmodulin-dependent protein kinase. Therefore, mechanism C would seem to be the most likely interpretation. This interpretation must, however, remain tentative at present. Furthermore a role for a calmodulin-inhibited protein phosphatase cannot be eliminated.

If protein phosphorylation is the mechanism underlying muscarinic receptor regulation then muscarinic receptor loss following agonist exposure should be accompanied by alterations in membrane protein phosphorylation. Such changes in the level of phosphorylation of 3 membrane proteins (of M_r 75 500, 67 000 and 62 000) have been demonstrated following exposure of primary cell cultures of rat cerebellum to the muscarinic agonist carbachol [26].

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