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Increased muscarinic receptor binding in heart membranes by an inhibitor of protein kinase C

Tony L. Creazzo and Robert W. Wrenn

Medical College of Georgia, Department of Anatomy, Augusta, GA 30912-2000, USA

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The number of muscarinic acetylcholine receptors (MAChRs), as detected by binding of [^{3}H]quinuclidinyl benzilate (QNB), was investigated under conditions which promote protein phosphorylation. Incubation of a crude heart membrane preparation in the presence of ATP/Mg^{2+} reduced MAChR number by 50%. Incubation with polymyxin B, an inhibitor of protein kinase C, blocked the effect of ATP/Mg^{2+} and increased MAChR number by 74%.

Muscarinic receptor; Phosphorylation; Acetylcholine; (Heart)

1. INTRODUCTION

Prolonged exposure of intact heart cells to cholinergic agonists results in a biphasic reduction in the number of muscarinic acetylcholine receptors (MAChRs) detectable by radiolabeled antagonists [1]. At least part of the apparent reduction of MAChRs is due to persistent binding of the unlabeled agonist. The loss of detectable MAChRs correlates with decreased sensitivity of the heart to cholinergic agonists. This phenomenon of MAChR desensitization is poorly understood. One possible mechanism is that desensitization results from phosphorylation of either the MAChR or a closely associated protein. In support of this hypothesis it has been reported that in vitro conditions which favor protein phosphorylation result in a loss of MAChRs [2–4]. Recently, Kwatra and Hosey [5] have found that treatment of hearts with the agonist carbachol leads to 10–12-fold increases in phosphorylation of the MAChR. In this report we show that polymyxin B (PMB), a selective inhibitor of protein kinase C [6], prevents the loss of MAChRs which results from incubation of crude heart membrane preparations under conditions which favor protein phosphorylation.

Correspondence address: T.L. Creazzo, Department of Anatomy, Medical College of Georgia, Augusta, GA 30912-2000, USA

2. MATERIALS AND METHODS

Fresh adult chicken hearts were a gift from Amick's Farm, Batesburg, SC. 1-[^{3}H]Quinuclidinyl benzilate (QNB) was purchased from Amersham/Searle. Atropine sulfate, adenine nucleotides and protein kinase A inhibitor were purchased from Sigma. Polymyxin B was purchased from United States Biochemical Corporation.

2.1. *Tissue preparation*

A crude membrane preparation was prepared in the following manner. The ventricles were coursely chopped, washed and homogenized with a Polytron in 100 mg tissue/ml of 50 mM Tris-HCl, pH 7.4 (buffer). The homogenate was centrifuged at 1000 × *g* for 10 min and the pellet discarded. The supernate was centrifuged at 21000 × *g* for 30 min. The supernate was discarded and the pellet resuspended in buffer to yield approx. 2 mg protein per ml. All steps were carried out at 4°C. The preparation was frozen at −80°C until used.

2.2. *Assay of [^{3}H]QNB binding*

[^{3}H]QNB binding was assayed and analyzed according to Cteazzo and Hartzell [7] except that non-specific binding was determined using 10 μM atropine sulfate. Assays were carried out overnight at room temperature in triplicate and terminated by filtration. Each assay tube contained 2 ml of buffer with

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500 pM [^{3}H]QNB (97% saturation) and 50 μl of homogenate (50–100 μg protein). Other additions are noted in the figure legends. Data from multiple experiments are expressed as means ± SE.

3. RESULTS

To test whether MAChR number is affected by conditions which would favor phosphorylation by protein kinases membranes were incubated in the presence of various adenine nucleotides (fig.1). Incubation with adenine nucleotides in the absence of Mg^{2+} produced either no effect or a slight elevation of MAChR binding to [^{3}H]QNB. In the presence of Mg^{2+}, ATP reduced the apparent number of MAChRs by 50%. In contrast, ADP and AMP reduced MAChRs only slightly when in the presence of Mg^{2+}. Similarly, the non-hydrolyzable ATP analog, AMP-PNP, had little effect. Scatchard analysis showed that the nucleotides or Mg^{2+} had no effect on [^{3}H]QNB-binding affinity (K_d = 11 pM, not shown). These results suggest that protein phosphorylation reduces the number of MAChRs that can be detected by [^{3}H]QNB.

To test further the possibility that phosphorylation reduces MAChR-binding sites, membranes were incubated with PMB, a specific inhibitor of protein kinase C (PKC) [6]. Fig.2A shows that PMB by itself increased the number of MAChRs in a dose-dependent fashion. In the presence of ATP and Mg^{2+}, PMB similarly increased MAChR sites

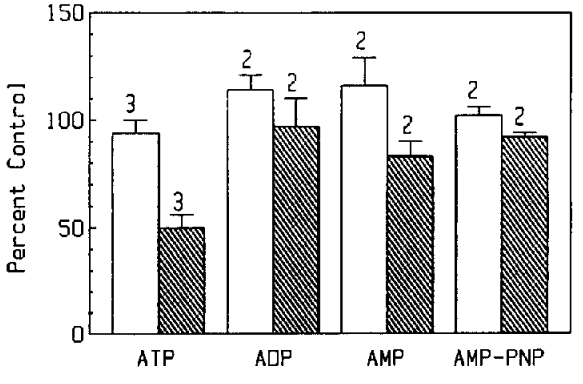

Fig.1. Effect of adenine nucleotides with and without Mg^{2+} on [^{3}H]QNB binding. (Open bars) Effects of 5 mM ATP, ADP and AMP and 1 mM AMP-PNP; (filled bars) effects of nucleotides plus 5 mM $MgCl_2$. The results were normalized to controls containing either no additions or 5 mM $MgCl_2$. The number of experiments is given above each bar.

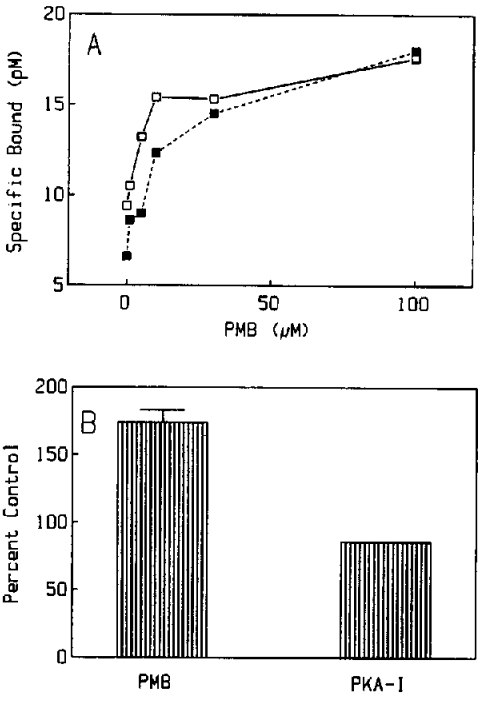

Fig.2. (A) PMB dose response. (Open squares) Incubation with PMB only; (filled squares) incubation with PMB plus 5 mM ATP and $MgCl_2$. (B) Effect of incubation of 100 μM PMB (n = 3) and 10 units/ml cAMP-dependent protein kinase inhibitor (PKA-I; n = 2) and 5 mM ATP and $MgCl_2$. The results were normalized to controls containing 5 mM ATP and Mg^{2+} without inhibitors.

in a dose-dependent manner though not as effectively as with PMB alone. Whether or not in the presence of ATP and Mg^{2+}, the maximum number of detectable MAChR-binding sites in the presence of 100 μM PMB was about 18 pM. Fig.2B indicates a 74% mean increase in the number of MAChRs with 100 μM PMB in the presence of ATP and Mg^{2+}. In contrast, an inhibitor of cAMP-dependent protein kinase [8] reduced MAChRs by 14%.

4. DISCUSSION

The results demonstrate that incubation of crude ventricle membranes with PMB, a specific inhibitor of PKC [6], substantially increases the number of MAChRs detectable with [^{3}H]QNB. Secondly, PMB inhibited the reduction of MAChRs that occurred in the presence of

ATP/Mg^{2+}. The cAMP-dependent protein kinase inhibitor (Walsh Inhibitor) [8], however, caused a slight reduction in MAChR number. These results suggest two possible mechanisms. The first is that PKC phosphorylation of the MAChR prevents binding of cholinergic ligands. The second possibility is that PKC phosphorylation activates a protein phosphatase which catalyzes dephosphorylation of the MAChR. This in turn would cause release of persistently bound acetylcholine, thereby allowing detection by [^{3}H]QNB. Under this scenario the observed loss of MAChRs in the presence of added ATP/Mg^{2+} (fig.1) may be due to some other protein kinase activity. The data presented here do not distinguish between these two possible mechanisms.

Though PKC activity is Ca^{2+} dependent it was not necessary to add Ca^{2+} in order to observe MAChR loss in ATP/Mg^{2+}. The addition of 200 μM Ca^{2+} to the assay did not enhance MAChR loss over ATP/Mg^{2+} alone (not shown). This is not surprising since contaminating Ca^{2+} is probably 10–20 μM or more. The K_m for Ca^{2+} stimulated PKC phosphorylation of histone H1 is 35 μM which decreases to 5–12 μM in the presence of phospholipid cofactors [9]. Also, the presence of other membrane lipid cofactors, such as diglycerides, lowers the K_m for Ca^{2+} to levels below 1 μM [10–11]. As already indicated by this discussion we cannot exclude the possibility that the reduction of MAChRs, observed in the presence of ATP/Mg^{2+}, was due to a kinase other than PKC.

It is interesting that PMB by itself increases MAChR binding without the addition of ATP/Mg^{2+}. One explanation is that PMB inhibits phosphorylation of the MAChR by the particulate PKC and ATP/Mg^{2+} already present in the crude membrane preparation. This is a reasonable assumption if the K_m for ATP/Mg^{2+} phosphorylation with PKC is in the low micromolar range; such as, for example, the K_m for PKC phosphorylation of histone H1 (4.4 μM) [12].

The data discussed in this report support the hypothesis that PKC-catalyzed protein phosphorylation plays a major role in MAChR desensitization and/or down regulation. Consistent with this hypothesis is a recent report indicating that PKC inhibits MAChR-modulated Ca^{2+} release and entry in human salivary cells [13].

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