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STIMULATION OF ACETYLCHOLINE RELEASE FROM GUINEA-PIG ILEAL SYNAPTOSOMES BY CYCLIC NUCLEOTIDES AND FORSKOLIN

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Abstract—Various agents which are known to affect intracellular levels of cAMP have been assessed for their ability to induce the release of [^{3}H]acetylcholine ([^{3}H]ACH) from a synaptosomal preparation derived from the guinea-pig ileum myenteric plexus. 8-Bromo-cAMP increased the release of [^{3}H]ACh above basal levels. While 8-bromo-cGMP also increased the release, this nucleotide was far less potent than 8-bromo-cAMP. Comparison of the release caused by the cyclic nucleotides to the release induced by the nicotinic agonist dimethylphenylpiperazinium (DMPP) suggested that there is some relationship, as yet undefined, between the 8-bromo-cAMP-induced and the DMPP-induced release, while no relationship was evident between the release induced by 8-bromo-cGMP and that caused by DMPP. The 8-bromo-cAMP-induced release was Ca^{2+}-dependent. Neither adenosine, clonidine, nor oxotremorine (all of which modulate the nicotinically-induced release) affected the 8-bromo-cAMP-induced release. The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine stimulated the release of [^{3}H]ACh as did the adenylate cyclase activator forskolin. The forskolin-induced release was not affected by adenosine, clonidine or oxotremorine. The ability of the modulators to block the nicotinically-induced release but not the release caused by the cyclic nucleotides indicates that the modulation of release evoked by nicotinic activity does not occur at a step involving protein phosphorylation.

Our interest in the mechanisms involved in the modulation of the release of [^{3}H]ACh† from nerve terminals led to the development of a synaptosomal preparation derived from the myenteric plexus of the guinea pig ileum [1]. We have shown that the nicotinic agonist DMPP evoked the release of [^{3}H]ACh from this preparation [2], and that this release was inhibited by stimulation of a muscarinic receptor [2], an adrenergic (α_2) receptor [3], and a purinergic receptor [4]: the purinergic receptor is of the A_1 type (H. Park and J. R. Cooper, unpublished observations).

Agents which increase the intracellular levels of cAMP have been reported to enhance the release of ACh from neurons of various peripheral preparations including that of the phrenic nerve-diaphragm [5], and the sciatic nerve-gastrocnemius muscle [6]. The membrane permeable cAMP analogue 8-bromo-cAMP has been shown to cause release from the longitudinal muscle-myenteric plexus preparation of the guinea pig ileum [7]. It should be noted, however, that in that study the phosphodiesterase inhibitor IBMX, which should raise intracellular levels of cAMP, had no effect on neurotransmitter release.

The present work was undertaken to determine whether cyclic nucleotides or agents which activate adenylate cyclase could release [^{3}H]ACh from synaptosomes derived from the myenteric plexus and, if so, could this release be modulated.

MATERIALS AND METHODS

Preparation of the P_2 fraction. Preparation of the crude synaptosomal fraction was as described elsewhere [1]. Briefly, the ileum from one Hartley guinea pig was removed and placed in KRB buffer consisting of NaCl (118 mM), KCl (4.7 mM), $CaCl_2$ (2.5 mM), $MgSO_4$ (1.2 mM), NaH_2PO_4 (1.2 mM), $NaHCO_3$ (25 mM) and dextrose (10 mM). The buffer (pH 7.4) was gassed with 95% O_2–5% CO_2. Longitudinal muscle strips [8] were minced and homogenized in 5 ml of 0.32 M sucrose containing 3 mM sodium phosphate buffer (pH 7.2). Following centrifugation at 1000 *g* for 10 min, the supernatant fraction was collected and centrifuged for 20 min at 17,000 *g*. The pellet (P_2) produced was used for the release studies.

Synthesis and release of [^{3}H]ACh. The tissue preparation containing the synaptosomes was incubated in 3 ml of KRB containing 3.0 μM [^{3}H]choline (5.0×10^3 Ci/mole) for 30 min at 37°. After centrifugation for 10 min at 5000 *g*, the pellet was washed in fresh KRB containing 10 μM eserine. The tissue was then suspended in 10 ml of the KRB plus eserine, and aliquots (200 μl) were added to 10 × 75 mm tubes which contained the appropriate drugs for a final volume of 220 μl. The tubes were incubated for 10 min at 37° under 95% O_2–5% CO_2. After returning the tubes to the ice bath, the tubes were

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† Abbreviations: ACh, acetylcholine; cAMP, adenosine 3′,5′-cyclic monophosphate; 8-bromo-cAMP, 8-bromo-adenosine 3′,5′-cyclic monophosphate; 8-bromo-cGMP, 8-bromo-guanosine 3′,5′-cyclic monophosphate; DMPP, dimethylphenylpiperazinium; EGTA ethylene glycol bis(β-aminoethyl ether) *N*,*N*′-tetracetic acid; IBMX, 3-isobutyl-1-methylanthine; and KRB, Krebs–Ringer bicarbonate.

centrifuged at 3600 *g* for 10 min, and the supernatant fractions were collected.

[^{3}H]ACh determination. The choline kinase-ion pair extraction method [9] was used to separate [^{3}H]ACH from [^{3}H]choline. The amount of [^{3}H]ACh released was evaluated as cpm/mg protein. Due to variations of release among preparations, similar to those found by O'Leary and Suszkiw [10], we report the various types of release as percentages of the basal release which was set at 100%. The basal release data in cpm/mg protein are given in each figure legend.

Statistics. Whenever appropriate, the analysis of variance was used to determine statistical significance. The figure legends contain the details.

Materials. The materials used included: 80×10^3 Ci/mole [methyl-^{3}H]choline chloride (New England Nuclear); physostigmine sulfate (ICN Pharmaceuticals); butyronitrile (Aldrich); glycylglycine (Calbiochem-Behring); adenosine (Boehringer Mannheim); and ATP, choline chloride, ATP:choline phosphotransferase (EC 2.7.1.32, choline kinase), DMPP iodide, 8-bromo-cAMP, 8-bromo-cGMP, IBMX, EGTA, and tetraphenylboron (Sigma).

RESULTS

Effects of cyclic nucleotides on release of [^{3}H]ACh. Increasing concentrations of 8-bromo-cAMP caused a marked increase in the release of [^{3}H]ACh from the guinea pig ileal myenteric plexus synaptosomes (Fig. 1A). The addition of DMPP (10 μM) produced an increased release of [^{3}H]ACh to levels above those induced by 8-bromo-cAMP alone. When 8-bromo-cGMP was evaluated, this cyclic nucleotide also stimulated the release of [^{3}H]ACh (Fig. 1B); however, 8-bromo-cGMP was far less potent than 8-bromo-cAMP. DMPP similarly stimulated the release above the levels produced by 8-bromo-cGMP.

The DMPP-induced release was the difference between the release in the presence of DMPP plus the cyclic nucleotide and the release caused by the cyclic nucleotide itself. The marked reduction in the DMPP-stimulated release as the concentration of 8-bromo-cAMP was increased (Fig. 1C) suggested that there was an interaction between the release caused by the two agents. The nature of the interaction is as yet undefined. In contrast, with 8-bromo-cGMP, the increase in release caused by DMPP was constant at all concentrations of the cyclic nucleotide which were tested, suggesting that no obvious interaction existed.

Effect of Ca^{2+} on the 8-bromo-cAMP-induced release. In the presence of 2.5 mM Ca^{2+}, 8-bromo-cAMP (4 mM) increased the release of [^{3}H]ACh from the synaptosomes (Fig. 2). In the absence of exogenous Ca^{2+} and the presence of 1 mM EGTA, both the basal and the 8-bromo-cAMP-stimulated release were reduced markedly. Thus, in contrast to the effect of 8-bromo-cAMP on protein phosphorylation which is independent of Ca^{2+} in brain slices [11], the release of ACh by this agent is dependent on the cation.

Effect of IBMX on the release of [^{3}H]ACh. The ability of IBMX, a phospodiesterase inhibitor [12, 13], to promote release was evaluated in the presence and absence of DMPP. IBMX stimulated the release of [^{3}H]ACh from the synaptosomes (Fig. 3) but not to the level produced by 8-bromo-cAMP. At lower concentrations of IBMX, DMPP stimulated the release above the level caused by IBMX alone. As IBMX was increased, however, the ability of DMPP to cause additional release was reduced.

Effects of neuromodulators on the 8-bromo-cAMP-stimulated release. Release by 8-bromo-cAMP was evaluated in the presence of adenosine (10 μM),

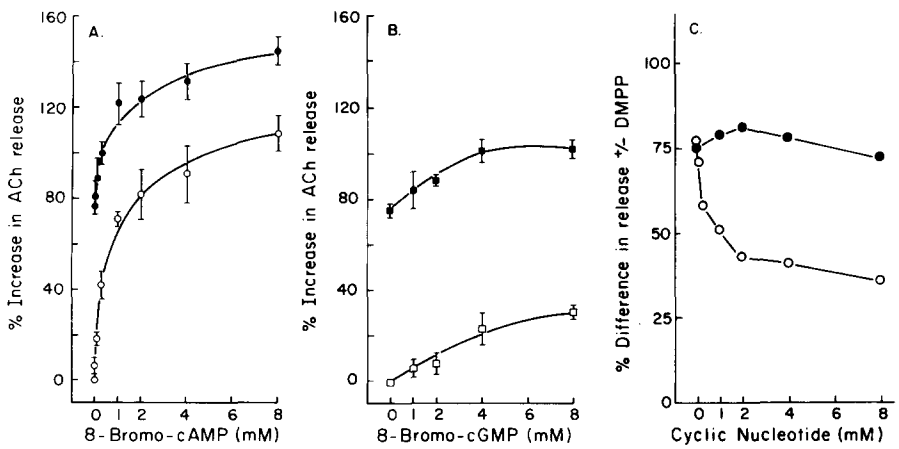

Fig. 1. Stimulation of release of [^{3}H]ACh from synaptosomes by cyclic nucleotides and DMPP (A) Effect of increasing concentrations of 8-bromo-cAMP in the absence (○) or presence (●) of DMPP. Release and determination of [^{3}H]ACh were as in Methods. The data are the means ± S.E.M. of three experiments. The basal release was 33,550 ± 6,490 cpm/mg protein (B) Effect of increasing concentrations of 8-bromo-cGMP in the absence (□) and presence (■) of DMPP. The basal release (mean ± S.E.M.) was 42,440 ± 10,320 cpm/mg protein. (C) The difference in release of [^{3}H]ACh in the presence and absence of DMPP with 8-bromo-cAMP (from A) (○) and with 8-bromo-cGMP (from B) (●).

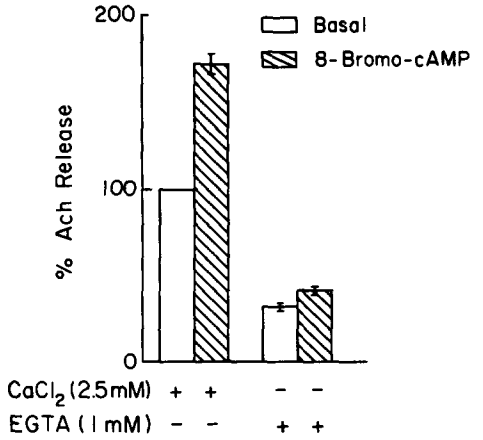

Fig. 2. Effect of Ca^{2+} on the basal and 8-bromo-cAMP-induced release of [^{3}H]ACh from the synaptosomes. All steps prior to the evaluation of release were carried out in KRB containing Ca^{2+}. The data are the means ± S.E.M. of three experiments. The basal release was 44,090 ± 190 cpm/mg protein. An analysis of variance on the raw data (cpm/mg protein) indicated that 8-bromo-cAMP significantly increased the release ($\alpha = 0.01$) and calcium significantly reduced both the basal and cyclic nucleotide-induced release ($\alpha = 0.01$).

clonidine (10 μM), and oxotremorine (100 μM). These concentrations were shown previously to be effective in inhibiting the DMPP-induced release [2–4]. As can be seen in Fig. 4A, these substances all caused reductions of the basal release and the total release in the presence of the nucleotide. However, when the 8-bromo-cAMP-stimulated release (the difference between the total release and the basal release) was determined, the 8-bromo-cAMP-induced release was not reduced significantly (Fig. 4B).

Effect of forskolin on the [^{3}H]ACh release. The addition of 50 μM forskolin, an activator of the catalytic subunit of adenylate cyclase [14], increased the release of [^{3}H]ACh by 60% when compared to the basal release (Fig. 5A). While each of the modulators, adenosine (10 μM), clonidine (10 μM) and oxotremorine (100 μM), reduced the basal release somewhat, the forskolin-induced release remained unchanged (Fig. 5B).

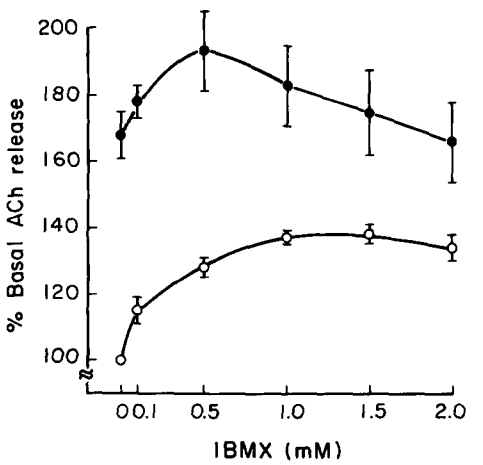

Fig. 3. Effect of IBMX on the release of [^{3}H]ACh in the absence (○) and presence (●) of DMPP. Release and determination of [^{3}H]ACh were as in Methods. The data are the means ± S.E.M. of three experiments. The basal release was 38,955 ± 4,487 cpm/mg protein.

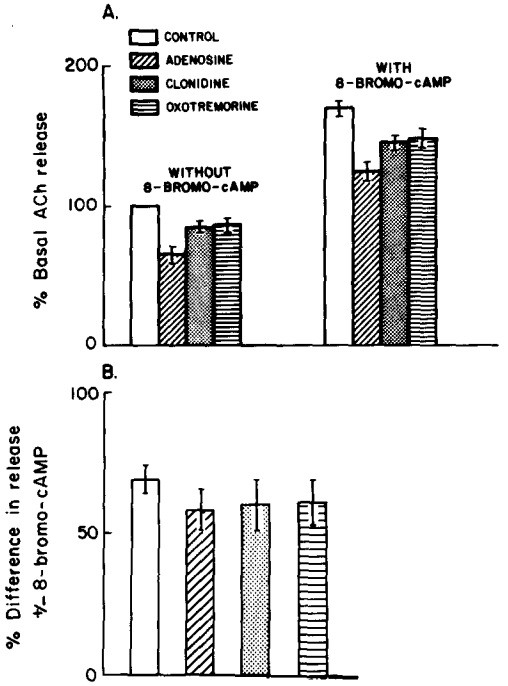

Fig. 4. (A) Effects of adenosine, clonidine, and oxotremorine on the release of [^{3}H]ACh in the presence or absence of 8-bromo-cAMP. The data are the means ± S.E.M. of three experiments. The basal release was 31, 107 ± 1,634 cpm/mg protein. (B) The difference in the release of [^{3}H]ACh in the presence or absence of 8-bromo-cAMP with adenosine, clonidine or oxotremorine (from A). An analysis of variance showed that the differences in release caused by the modulators were not significant.

DISCUSSION

Alberts and Stjarne [7] have demonstrated that the release of ACh from the guinea pig ileum myenteric plexus by electrical field stimulation is increased by 55% in the presence of 0.5 mM 8-bromo-cAMP. Our interest in the possibility that cAMP might act as a second messenger in the DMPP-stimulated release of [^{3}H]ACh from the myenteric plexus synaptosomes led us to evaluate the ability of 8-bromo-cAMP to cause release of [^{3}H]ACh from this tissue. This membrane permeable cAMP analogue induced release of [^{3}H]ACh at concentrations up to 8 mM. Concentrations of this magnitude were evaluated based on the findings of Forn and Greengard [11] that these concentrations of 8-bromo-cAMP maximally stimulated the phosphorylation of membrane proteins, which may be involved in specific synaptosomal functions [15].

DMPP, which maximally stimulates the nicotinically-induced release of [^{3}H]ACh [2], caused

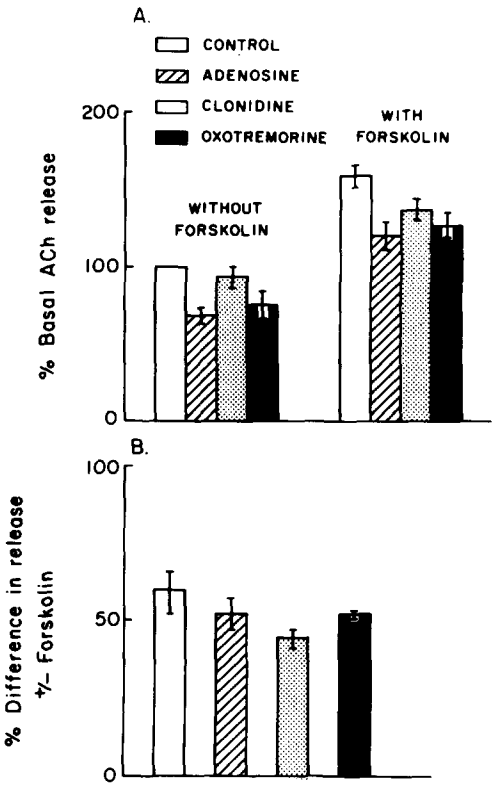

Fig. 5. (A) Effects of adenosine, clonidine, and oxotremorine on the release of [^{3}H]ACh in the presence or absence of 50 μM forskolin. The data are the means ± S.E.M. of three experiments. The basal release was 41,913 ± 8,715 cpm/mg protein. (B) The difference in the release of [^{3}H]ACh in the presence or absence of forskolin with adenosine, clonidine or oxotremorine (from A). An analysis of variance showed that the differences in release caused by the modulators were not significant.

an additional increase in [^{3}H]ACh release at each concentration of 8-bromo-cAMP used. At the higher concentrations of 8-bromo-cAMP, however, the nicotinically-induced increase in release was much reduced. This suggests that there may be some relationship between the release caused by 8-bromo-cAMP and that caused by DMPP. For example, the same pool of neurotransmitter or the same mechanism of release could be involved. The explanation for this possible relationship, however, remains unknown.

At concentrations up to 8 mM, 8-bromo-cGMP caused release of [^{3}H]ACh from the synaptosomes. This release, however, was much smaller than that caused by 8-bromo-cAMP. While DMPP also increased the release in the presence of the cGMP analogue, this nicotinically-induced release was constant at all concentrations of 8-bromo-cGMP tested. This suggested that there was no direct relationship between the two types of release. Alberts and Stjarne [7] reported no effect of 8-bromo-cGMP on the release of [^{3}H]ACh. While the specific reason for the different findings in these two studies is not clear, Alberts and Stjarne were using only 0.5 to 1.0 mM 8-bromo-cGMP, and the tissue was the intact longitudinal muscle-myenteric plexus preparation.

The presence of calcium is known to be required for the release of neurotransmitter from nerve terminals [16]. We have shown previously that the basal and the DMPP-induced release from ileal synaptosomes were calcium dependent [2]. We therefore wanted to determine whether the release caused by 8-bromo-cAMP required the presence of calcium. Calcium was required for the 8-bromo-cAMP-induced release in contrast to the calcium-independent effect of the cyclic nucleotide in promoting protein phosphorylation. This observation implies that, if protein kinase activity is involved in the release process, another step requiring the presence of extracellular Ca^{2+} is also involved.

Further support of a second messenger role for cyclic nucleotides is provided by experiments with IBMX, a phosphodiesterase inhibitor [12, 13]. In our study, IBMX stimulated the release of [^{3}H]ACh from the synaptosomes, suggesting that 8-bromo-cAMP may have been acting through a cAMP-dependent mechanism. On the other hand, Alberts and Stjarne [7] did not find an increase in release of ACh from the guinea pig myenteric plexus with IBMX. While the tissue preparation and the experimental conditions of the present study and that of Alberts and Stjarne were different, the basic explanation for the disagreement in findings is unclear.

Adenosine, clonidine, and oxotremorine previously were reported to reduce the DMPP-induced release of [^{3}H]ACh from the guinea pig ileal synaptosomes [2–4]. These modulators, however, did not affect the 8-bromo-cAMP-induced release. The results indicated that the inhibition of the nicotinically-induced release caused by adenosine, clonidine and oxotremorine must act at some point prior to the point of cyclic AMP activity and subsequent protein phosphorylation.

Similarly, forskolin, which directly stimulates the catalytic subunit of adenylate cyclase causing an increase of intracellular cAMP [17, 18], stimulated the release of [^{3}H]ACh. Neither adenosine, clonidine nor oxotremorine, however, reduced this forskolin-stimulated [^{3}H]ACH release. If DMPP, in fact, requires cAMP as a second messenger for the release of [^{3}H]ACh, then the inability of the modulators of the DMPP-induced release to affect the forskolin-induced release implies that the modulation must occur at some point prior to the actual production of cAMP by adenylate cyclase. Whether the modulation is a direct action on calcium channels or at the receptor level is as yet unknown.

This paper has demonstrated that [^{3}H]ACh can be released from guinea pig ileal myenteric plexus synaptosomes by agents (IBMX, forskolin) which raise intracellular cAMP levels and by the direct action of the membrane permeable cAMP analogue 8-bromo-cAMP. Since the cAMP cascade leads to protein phosphorylation which, in turn, implicates protein kinase activity, our results suggest that a cAMP-dependent protein kinase is involved. On the other hand, we have observed (unpublished) that trifluoperazine inhibits the DMPP-induced release of ACh in our preparation. This observation adds an additional level of complexity to the release mech-

anism, with the implication of calmodulin, perhaps via an activation of adenylate cyclase [17]. At any rate, regardless of the protein phosphorylation mechanism, it is clear from our results that this mechanism is not involved in modulatory activity since our previously described modulators exert their inhibitory activity only when ACh release is evoked by DMPP and not by the agents which lead to protein phosphorylation.

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