Acetylcholine stimulates phosphatidylinositol turnover at nicotinic receptors of cultured myotubes

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Acetylcholine treatment of [^{3}H]inositol pre-labelled cultured chick embryo myotubes results in the stimulation of phosphatidylinositol breakdown, as shown by the measurement of inositol-1-phosphate accumulating in the presence of lithium. The described effect is dependent on agonist concentration and incubation time, and is inhibited by tubocurarine and α-bungarotoxin. The activation of phosphatidylinositol breakdown by acetylcholine at extrajunctional nicotinic receptors is likely to be involved in the modulation of the functional activity of the receptor.

Acetylcholine *Nicotinic receptor* *Phosphoinositide* *Cultured myotube* *Receptor modulation*

1. INTRODUCTION

Several agonists which bind to specific receptors on the cell membrane have been shown, in recent years, to activate phosphatidylinositol (PtdI) breakdown, an effect that initiates an amplified cascade of intracellular events (protein kinase C activation, release of calcium from intracellular stores and, secondarily, involvement of the prostaglandins and cyclic nucleotides systems) leading to physiological effects (review [1,2]).

Multinucleated myotubes, formed in culture by chick embryo myoblasts, express acetylcholine (ACh) receptors on their entire surface (extrajunctional receptors) and respond to ACh by membrane depolarization. Their receptors, which have been shown to be nicotinic in nature [3,4], become desensitized when continuously exposed to the transmitter. The molecular mechanism underlying such desensitization is not well understood. In analogy with other systems, phosphorylation of the receptor itself could play a role in such a regulatory mechanism [5,6], and it is known that the α and δ subunits of *Torpedo* nicotinic ACh receptors may be phosphorylated by protein kinase C [7].

We have recently shown that agents known to activate protein kinase C, such as the tumor promoter 12-*O*-tetradecanoyl phorbol-13-acetate and diacylglycerol, reduce ACh sensitivity and increase the rate of desensitization in chick embryo cultured myotubes [8]. It was therefore of interest to investigate whether ACh was capable of inducing PtdI breakdown at the nicotinic receptor, an event that may relate the function of ACh receptor channels to protein kinase C activity.

2. MATERIALS AND METHODS

Chick embryo myogenic cells were prepared and cultured as described [9] in 6 cm tissue culture dishes. Cultures consisting of multinucleated myotubes, virtually devoid of fibroblasts, were obtained using cytosine arabinoside (2.5 μg/ml) from 36 to 66 h of culture, as described [10]. [1,2(n)-^{3}H]Inositol (NEN, spec. act. 50 Ci/mmol), 5 μCi/ml, was added on the third day of culture. On the fourth day of culture, the cells were rinsed

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5 times over a period of 90 min with basal medium (without serum and embryo extract) and equilibrated for 15 min at 37°C. 20 mM LiCl was added to the cultures 10 min prior to the beginning of ACh treatment. The cultures were then incubated with ACh at the concentrations and for the times indicated in the figure legends. At the end of the incubation, the medium was rapidly removed, the cultures transferred on ice and 1 ml of 10% trichloroacetic acid was added to each dish. After 30 min at 4°C, the supernatant was removed and the cultures rinsed with H_2O. The trichloroacetic acid and H_2O extracts were combined, centrifuged at 2000 rpm for 10 min, and the supernatants were extracted 7 times with water saturated diethyl ether, neutralized by the addition of 1 ml of 0.1 M phosphate buffer, pH 7.0, and the volume of each sample was brought to 8 ml with H_2O. The samples were poured onto columns containing 1 ml of Dowex 1X8-200 resin (Sigma) and elution was performed as described by Berridge [11]. Recovery of radioactivity from the columns was constantly higher than 90%, approx. 70–80% being represented by free inositol, 1.5% by glycerophosphoinositol, 8–20% by inositol-1-phosphate (IP_1), 5% by inositol-1,4-diphosphate (IP_2) and 5% by inositol-1,4,5-triphosphate (IP_3), as identified on the basis of their elution properties [11,12].

For the experiments on the effect of ACh and lithium on PtdI labelling described in table 1, unlabelled myotube cultures were pre-incubated with or without 20 mM LiCl for 10 min. At time 0, the cultures received 5 μCi/ml [^{3}H]inositol, with or without 20 μM ACh, as appropriate. After 60 min, the medium was rapidly removed, and the cells were extracted with chloroform/methanol/conc. HCl (200:100:1). The lower phase was applied to silica gel G thin layer plates (Merck), along with standard authentic phospholipids [13]. The chromatography was developed with chloroform/methanol/conc. acetic acid/water (25:15:2:1) and the standards were visualized by spraying the plate with 40% (v/v) H_2SO_4 and incubating at 120°C for 30 min [13]. Each lane was scraped in 1 cm sections, eluted with chloroform/methanol/conc. HCl (100:50:1) and counted with Permafluor (Packard). Approx. 20% of the radioactivity was recovered at the origin, 40–60% comigrated with standard PtdI (R_f = 0.47), and two minor peaks had R_f = 0.20 and 0.90, respectively.

3. RESULTS AND DISCUSSION

Incubation of [^{3}H]inositol pre-labelled chick embryo myotubes with 20 μM ACh, in the presence of 20 mM LiCl, results in accumulation of labelled IP_1, increasing with incubation time over that of controls (fig.1). IP_2 and IP_3 levels are not significantly influenced by ACh treatment over the time period examined, as expected on the basis of

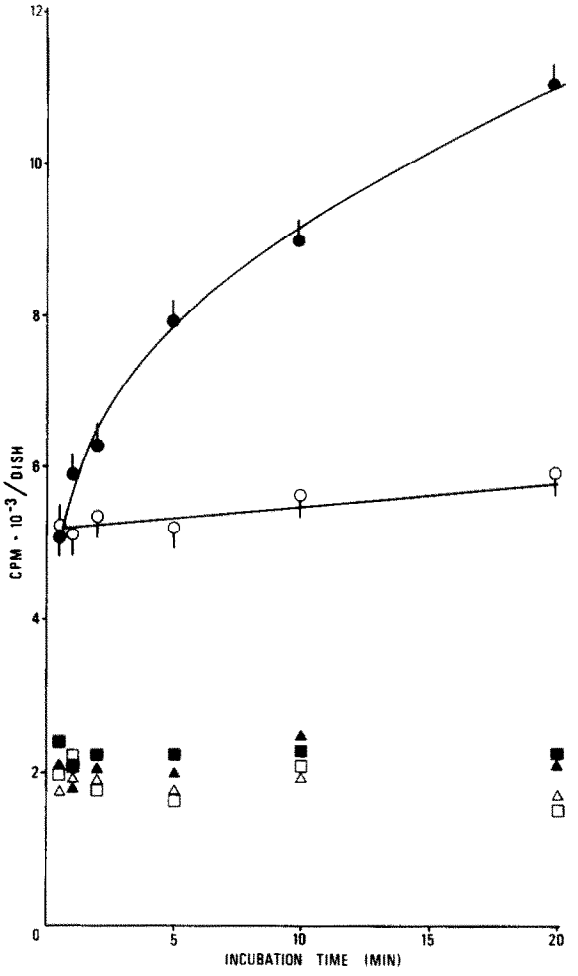

Fig.1. Time course of the effect of ACh on the levels of inositol phosphates in cultured myotubes. All samples were incubated with 20 mM LiCl 10 min prior to the beginning of ACh treatment. Symbols: IP_1 (●, ○), IP_2 (▲, △), IP_3 (■, □); solid symbols, ACh treated samples; empty symbols, control samples.

the specificity of the inhibitory effect of lithium for inositol-1-phosphatase [14]. The accumulation of IP_1 is rapid during the first minutes of ACh treatment, then the rate of accumulation slightly declines, suggesting that the rate of PtdI breakdown may decrease after the first few minutes of ACh treatment. The time course of IP_1 accumulation is fully compatible with the time course of the depolarization induced by similar concentrations of ACh in chick embryo myotubes [4,8].

A dose-response curve of the effect of ACh on PtdI breakdown (measured by IP_1 accumulation in the presence of lithium) is shown in fig.2. Significant stimulation of IP_1 accumulation occurs at ACh concentrations as low as 10^{-7} M, with a steep increase up to about 20 μM. Higher ACh concentrations do not further increase IP_1 accumulation. Both 100 nM α-bungarotoxin and 150 μM tubocurarine inhibit almost completely the effect of 20 μM ACh (fig.2), without modifying the control level of any inositol phosphate.

IP_1 accumulation represents a sensitive method to evaluate PtdI breakdown, however the effect of ACh on PtdI turnover has also been studied using a different experimental approach, as shown in table 1. If unlabelled myotubes are incubated for 60 min with ACh and, during the same period, with [^{3}H]inositol, the labelling of PtdI is stimulated by ACh treatment of the cultures. This result is reasonable on the basis of increased turnover of the phospholipid. Interestingly, also lithium alone increases PtdI labelling, presumably because it prevents the reutilization of the IP_1 deriving from the hydrolysis of preexisting, unlabelled PtdI.

Our results indicate that increased PtdI turnover is induced by physiological concentrations of ACh [15] at extrajunctional nicotinic receptors in cultured chick embryo myotubes. It has been shown that ACh induces PtdI breakdown at muscarinic receptors [16,17]. However, the physiological role of the stimulation of PtdI turnover might be different for these two types of ACh receptors. In fact, some of the agents known to activate PtdI breakdown upon interaction with their specific receptor (e.g. α-adrenergic agonists, vasopressin, bombesin, muscarinic agonists) probably utilize this regulatory system as the main route to achieve their respective intracellular effects [1]. For other agents (e.g. EGF, insulin, somatomedin C) [16,18–20] the activation of PtdI turnover may represent a collateral effect, probably not involved (or marginally involved) in the generation of their physiological responses, but responsible for the modulatory action on the func-

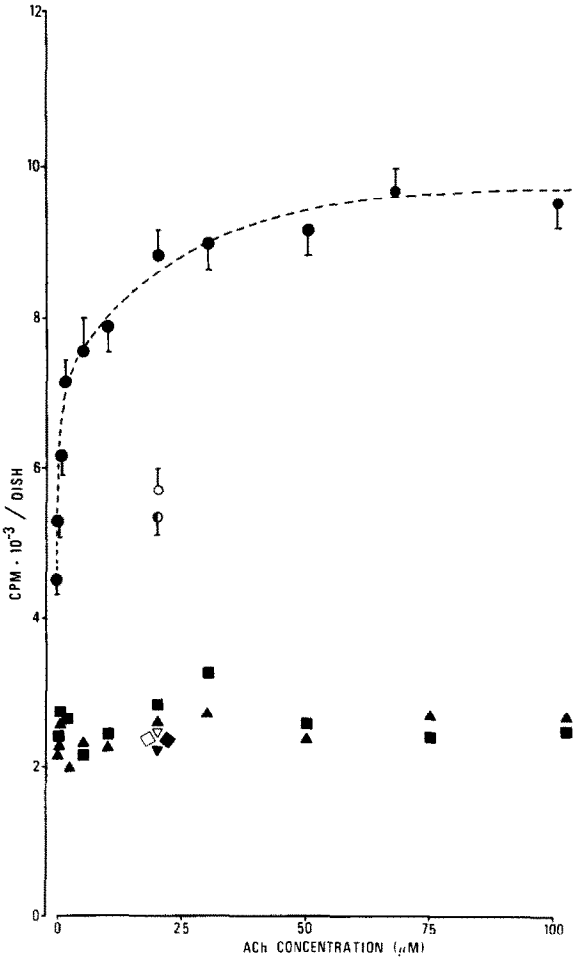

Fig.2. Concentration dependency of the effect of ACh on the levels of inositol phosphates in cultured myotubes. All samples were incubated with 20 mM LiCl 10 min prior to the beginning of a 15 min ACh treatment. 100 nM α-bungarotoxin or 150 μM tubocurarine were added 10 min before LiCl to cultures to be treated with 0 or 20 μM ACh. Neither inhibitor had significant effect on the control level of any inositol phosphate. Symbols: IP_1 (●); IP_2 (▲); IP_3 (■); α-bungarotoxin treated cells, IP_1 (○), IP_2 (▽) and IP_3 (◇); tubocurarine treated cells, IP_1 (◐), IP_2 (▼) and IP_3 (◆).

Table 1

Effect of lithium and acetylcholine on the labelling of phosphatidylinositol

Treatment	PtdI (cpm/dish)	%
Control	197 ± 21	100
20 mM LiCl	329 ± 40	167
20 μM ACh	411 ± 33	208
20 mM LiCl + 20 μM ACh	536 ± 47	272

For experimental details, see section 2

tional activity of their specific membrane receptors [6,21].

As mentioned above, protein kinase C may phosphorylate the α and δ subunits of the nicotinic ACh receptor [7]. An attractive hypothesis is that the neurotransmitter ACh activates protein kinase C via PtdI breakdown stimulation. Our previous results showing that the desensitization of ACh receptors is increased by protein kinase C activators [8] are in favour of this hypothesis. The phosphorylation of ACh receptor by protein kinase C might modulate membrane conductance and the kinetic properties of single ACh receptor channels, and might also be involved in the receptor desensitization. In addition, release of intracellular calcium could be induced by IP_3, upon activation of PtdI breakdown [1,2]. This event, alone or synergistically with protein kinase C activation, could play a role in ACh receptor desensitization [22].

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