Pergamon

Biochemical Pharmacology, Vol. 50, No. 5, pp. 647–654, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)00179-4

ETHANOL INHIBITS THE PEAK OF MUSCARINIC RECEPTOR-STIMULATED FORMATION OF INOSITOL 1,4,5-TRISPHOSPHATE IN NEUROBLASTOMA SH-SY5Y CELLS

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(*Received* 9 *August* 1995; *accepted* 10 *April* 1995)

Abstract—The effect of ethanol on muscarinic receptor-stimulated formation of inositol 1,4,5-trisphosphate was studied in human neuroblastoma SH-SY5Y cells. Stimulation with carbachol induced a biphasic increase of inositol 1,4,5-trisphosphate with an initial peak after 10 sec declining to a plateau phase of elevation above basal levels, which was sustained for at least 5 min in the presence of agonist. The peak, but not the plateau phase, was concentration-dependently decreased by exposure to ethanol. Maximal inhibition was obtained within 30 sec of exposure to ethanol. Ethanol caused an increase in the EC_{50} value of carbachol for the initial rate of inositol 1,4,5-trisphosphate formation, measured after 10 sec of stimulation, from 98 μM in the absence to 196 μM in the presence of 100 mM ethanol. The potencies of pirenzepine and hexahydro-sila-difenidol hydrochloride for inhibiting [^{3}H]quinuclidinyl benzilate binding and inositol 1,4,5-trisphophate formation suggest that both phases are mediated via the muscarinic M_1 receptor. Phorbol 12-myristate 13-acetate inhibited both phases of inositol 1,4,5-trisphosphate formation, whereas okadaic acid and modulators of cAMP-dependent protein kinase were without any effect. There was no inhibitory effect of ethanol when protein kinase C was inhibited by H7 and calphostin C, indicating that the ethanol effect is dependent on protein kinase C activity.

Key words: muscarinic-receptor; inositol 1,4,5-trisphosphate-formation; ethanol; protein kinase C; neuroblastoma SH-SY5Y cells

Receptor-stimulated signal transduction in the nervous system has been shown to be affected by acute exposure to ethanol. With regard to the phospholipase C transduction system, ethanol has been reported to exert differential effects that are receptor and/or cell specific [1–5]. These studies have measured the total accumulation of [^{3}H]-inositol phosphates, usually in the presence of Li^+. The results thereby reflect the mean of the stimulated phospholipase C activity for an extended period of time usually without direct measurement of the initial rate of I(1,4,5)P_3‡ generation.

More recent studies measuring either endogenous levels of I(1,4,5)P_3 by a binding assay or the radioactivity of this metabolite in [^{3}H]-inositol labelled cells have revealed different temporal patterns in receptor-stimulated I(1,4,5)P_3 increase. In human neuroblastoma SH-SY5Y cells, stimulation with carbachol elicits a rapid and transient peak of I(1,4,5)P_3 levels, followed by a decline to a lower level, although higher than basal, where they remain as long as the agonist is present [6].

The aim of this study was to elucidate whether exposure to ethanol will affect the two phases of carbachol-stimulated I(1,4,5)P_3 increase in SH-SY5Y cells and to relate the ethanol effect to the different mechanisms regulating the two phases of I(1,4,5)P_3 formation. The data demonstrate that ethanol selectively affects the initial rate of I(1,4,5)P_3 formation, but does not influence steady-state elevation induced by carbachol. This effect of ethanol is mediated by a protein kinase C-dependent mechanism.

MATERIALS AND METHODS

Materials. SH-SY5Y cells were a gift from Dr S Påhlman (Department of Pathology, Uppsala University, Sweden). Cell culture solutions and okadaic acid were from Gibco. Cell culture dishes were from Costar. [^{3}H]inositol 1,4,5-trisphosphate (15–30 Ci/mmol) and [^{3}H]quinuclidinyl benzilate (QNB, 43 Ci/mmol) were from New England Nuclear. Calphostin C, carbachol, CPTcAMP, PMA, PZ, and staurosporine were from Sigma. HHSD and H7 were from Research Biochemicals Inc. RpcAMPS was from Biolog (Bremen, Germany). Ready safe scintillation cocktail was from Beckman. All other chemicals were from Merck.

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‡ Abbreviations: CCh, carbachol; CPTcAMP, 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate; H7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine; HHSD, hexahydro-sila-difenidol hydrochloride; I(1,4,5)P_3, inositol 1,4,5-trisphosphate; PMA, phorbol 12-myristate 13-acetate; RpcAMPS, Rp-isomer of adenosine-3′,5′-cyclic monophosphothioate; TCA, trichloroacetic acid; PZ, pirenzepine.

Culture and stimulation of SH-SY5Y cells. SH-SY5Y cells were cultivated in Eagle's minimal essential medium with L-glutamine and Earl's salts supplemented with 10% foetal calf serum, 100 IU/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere with 5% CO_2. Cells were seeded at 350,000 cells/60 mm dish and allowed to grow for 1 week after passage when they reached confluency. The medium was changed once during this time. Cells used were from passages 50–70.

Fifteen minutes prior to stimulation, the complete medium was exchanged for Eagle's minimal essential medium without any further supplements. All incubations were performed at 37°. Muscarinic antagonists were added 2 min prior to stimulation and other drugs were added 5 min prior to stimulation. Stimulation was interrupted by aspiring the medium and adding 0.5 mL 0.5 M TCA.

Analysis of endogenous I(1,4,5)P_3. After termination of stimulation, cells were scraped and centrifuged at 2000 × *g* for 15 min at 4°. The pellet was mixed in 250 μL 1 M NaOH and stored for protein analysis [7]. The supernatant was extracted four times with 1.25 ml water-saturated diethylether. The cell extract was neutralized by adding 20 μL 500 mM Tris-HCl, pH 8.4. A receptor binding assay developed by Bredt *et al.* [8] was used for determination of endogenous I(1,4,5)P_3 levels. Bovine cerebellar membranes (rich in I(1,4,5)P_3 binding sites) were prepared by homogenizing bovine cerebella in cold buffer A (50 mM Tris, 1 mM EDTA, 1 mM 2-mercaptoethanol, pH 7.7) and washing them by repeated centrifugations at 15,000 × *g* for 15 min. In the binding assay, cerebellar membranes (60 μg/tube), diluted in buffer A at pH 8.6, were added together with 0.2 pmol [^{3}H]-I(1,4,5)P_3 (15 cpm/fmol) to Eppendorf microfuge tubes. Standard solutions of I(1,4,5)P_3 or neutralized cell extracts were added to a final volume of 275 μL/tube. After 10 min on ice, displaced [^{3}H]I(1,4,5)P_3 was removed by centrifuging tubes 10,000 × *g* for 5 min in a Beckman microfuge and aspiring the supernatant. The pellet was solubilized in 500 μL water and taken for scintillation counting. A standard curve of 1–100 nM I(1,4,5)P_3 was used. Addition of exogenous I(1,4,5)P_3 (1–100 nM) to cell homogenates demonstrated that the recovery was 117 ± 27% (mean ± SD).

Binding of [^{3}H]QNB to intact SH-SY5Y cells. SH-SY5Y cells were detached from the dish by washing each 60 mm dish with 1 ml HEPES-buffered medium. The cells were pooled and 400 μL of the cell suspension added to 1.5 mL Eppendorf tubes containing [^{3}H]QNB and muscarinic antagonists. Following incubation in a shaking water-bath for 2 hr at 37°, unbound [^{3}H]QNB was removed by centrifugation for 1 min in a microfuge followed by aspiration of the supernatant. The pellet containing the SH-SY5Y cells was solubilized in 500 μL water and taken to scintillation counting. Non-specific binding was assayed in the presence of 10 μM atropine.

Data analysis. EC_{50} and IC_{50} values and maximal responses were calculated with the curve-fitting function in the SigmaPlot™ 1.02 for Windows computer program. Values were fitted to the hyperbolic function $y = a/(1+(b/x)^n)$ where a is the maximal response, b is the drug concentration that induces 50% of maximal effect, n is the slope factor, and y the response induced by the concentration x of the drug. The use of a two-site fit was assessed by comparing the one-site (without slope factor) and two-site fits with the F-test. In the functional assay, the IC_{50} value was converted to K_b with the equation $K_b = IC_{50}/((2+([A]/EC_{50})^n)^{1/n} - 1)$, with A being the concentration of the agonist used and n the slope factor for the agonist concentration–response curve [9, 10]. In the binding assays, the IC_{50} was converted to $K_{0.5}$ values using the Cheng–Prusoff equation. All means of EC_{50}, IC_{50}, K_b and $K_{0.5}$ values are geometric means and statistical analyses were performed using the logarithms of the pharmacological constants. Student's t-test was used for statistical analyses comparing two separate groups.

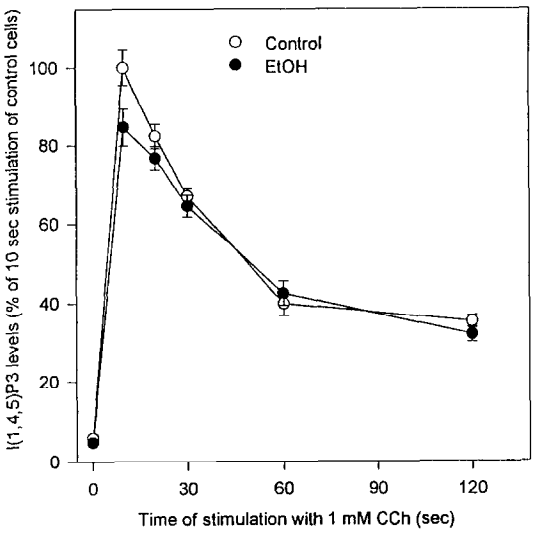

Fig. 1. Time-course of the carbachol-induced (1 mM) increase in I(1,4,5)P_3 levels in SH-SY5Y cells in the presence (filled circles) and absence (hollow circles) of 100 mM ethanol. Ethanol was added 2 min before stimulation. Stimulated levels after 10 sec in the absence of ethanol were 341 ± 23 pmol/mg protein. Data are expressed as the percentage of the amount of I(1,4,5)P_3 in control cells after stimulation for 10 sec with 1 mM carbachol and are mean ± SEM from three experiments (N = 6–9).

RESULTS

Ethanol effects on carbachol-induced I(1,4,5)P_3 formation

Stimulation of SH-SY5Y cells with 1 mM carbachol induced a biphasic increase in I(1,4,5)P_3 with an initial peak reaching a maximum after 10 sec. Thereafter, the amounts declined and leveled off after 60 sec, remaining elevated at approximately 40% of the peak level. The peak of carbachol-stimulated I(1,4,5)P_3 formation was inhibited by pretreatment with 100 mM ethanol for 2 min whereas

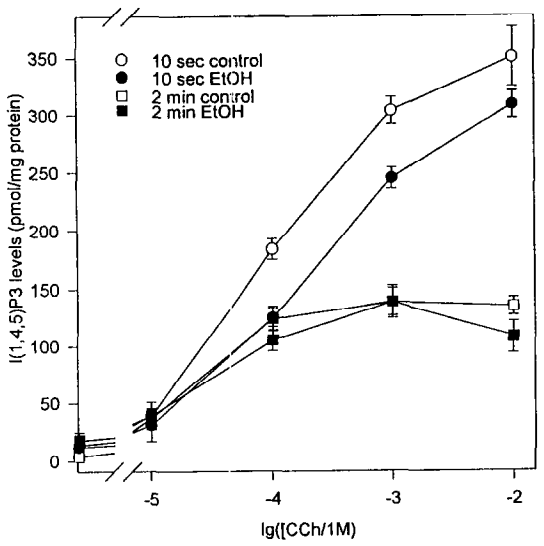

Fig. 2. Concentration–response curves for carbachol-induced increases in $I(1,4,5)P_3$ levels in SH-SY5Y cells, monitored after 10 sec (circles) or 2 min of stimulation (squares) in the absence (hollow symbols) or presence (filled symbols) of 100 mM ethanol. Ethanol was added 2 min prior to stimulation. Data are mean ± SEM of three experiments carried out in triplicate.

the plateau level was insensitive to ethanol exposure (Fig. 1).

The effect of ethanol was further illustrated in experiments where SH-SY5Y cells were stimulated with increasing concentrations of carbachol for 10 sec or 2 min (Fig. 2). The EC_{50} for carbachol was 98 μM for the $I(1,4,5)P_3$ increase after 10 sec and 50 μM for the levels after 2 min (Table 1). Application of 100 mM ethanol decreased $I(1,4,5)P_3$ levels after 10 sec of carbachol stimulation but did not affect the 2-min stimulation. Significant effects of ethanol on stimulated $I(1,4,5)P_3$ levels were observed when 100 μM carbachol (184 ± 9 pmol/mg protein in the absence and 125 ± 12 pmol/mg protein in the presence of ethanol, $P < 0.01$, $N = 9$) or 1 mM carbachol (304 ± 12 pmol/mg protein in the absence and 245 ± 17 in the presence of ethanol, $P < 0.05$, $N = 9$) was used. Basal $I(1,4,5)P_3$ levels were not affected by ethanol exposure (14 ± 4 pmol/mg protein in control cells and 12 ± 3 pmol/mg in ethanol exposed cells). A decreased potency of carbachol was observed in the presence of ethanol as indicated by the increase in the EC_{50} value upon ethanol exposure (Table 1). The effect of ethanol on the EC_{50} at the 10 sec time point was significant ($P < 0.05$) when a *t*-test analysis was performed on the pD2 values.

The effect of different incubation times and concentrations of ethanol pretreatment was investigated (Fig. 3). As shown in Fig. 3(A), 30 sec of ethanol pretreatment was sufficient to induce a maximal effect of 20% inhibition of $I(1,4,5)P_3$ levels induced by a 10 sec stimulation with 1 mM carbachol. Pretreatment with ethanol for longer time periods (up to 10 min) did not further enhance the effect. Pretreatment with varying concentrations of ethanol for 5 min caused a concentration-dependent inhibition of carbachol-induced $I(1,4,5)P_3$ formation (Fig. 3B). Although 100 mM ethanol was necessary to obtain a significant effect, a tendency toward inhibition could be observed at lower concentrations.

Effects of different muscarinic antagonists

The selective effect of ethanol on the peak of $I(1,4,5)P_3$ could be due to the two phases being mediated via separate muscarinic receptors. The muscarinic antagonists pirenzepine and HHSD were applied in an attempt to elucidate which muscarinic receptors the different phases of $I(1,4,5)P_3$ increase were mediated through (Fig. 4). The antagonists concentration-dependently inhibited the peak (as measured after 10 sec of stimulation) as well as the plateau (measured after 2 min of stimulation) of $I(1,4,5)P_3$ increase. The IC_{50} and K_b values of the antagonists are summarized in Table 2. The potency of pirenzepine suggests that the responses are mediated via muscarinic M_1 receptors. To confirm the presence of M_1 receptors on these cells, the binding of [^{3}H]QNB and the displacement of this substance by PZ and HHSD were assayed with intact SH-SY5Y cells (Fig. 5). The K_d of [^{3}H]QNB was 28 pM ($pK_d = 10.6 \pm 0.04$) and the maximal binding

Table 1. Carbachol-stimulated formation of $I(1,4,5)P_3$ in SH-SY5Y cells*

	10 sec		2 min	
	pD2	EC_{50}	pD2	EC_{50}
pD2 and EC_{50} values of carbachol				
Control	4.01 ± 0.06	98 μM	4.30 ± 0.18	50 μM
EtOH (100 mM)	3.70 ± 0.08†	196 μM	4.26 ± 0.09	55 μM
Maximal response (pmol $I(1,4,5)P_3$/mg protein)				
Control	334 ± 24		160 ± 6	
EtOH (100 mM)	290 ± 40		125 ± 15	

* The pD2 and the maximal response are expressed as algebraic mean ± SEM. The EC_{50} value is expressed as the geometric mean. $N = 3$ for all values.

† $P < 0.05$ compared to no ethanol.

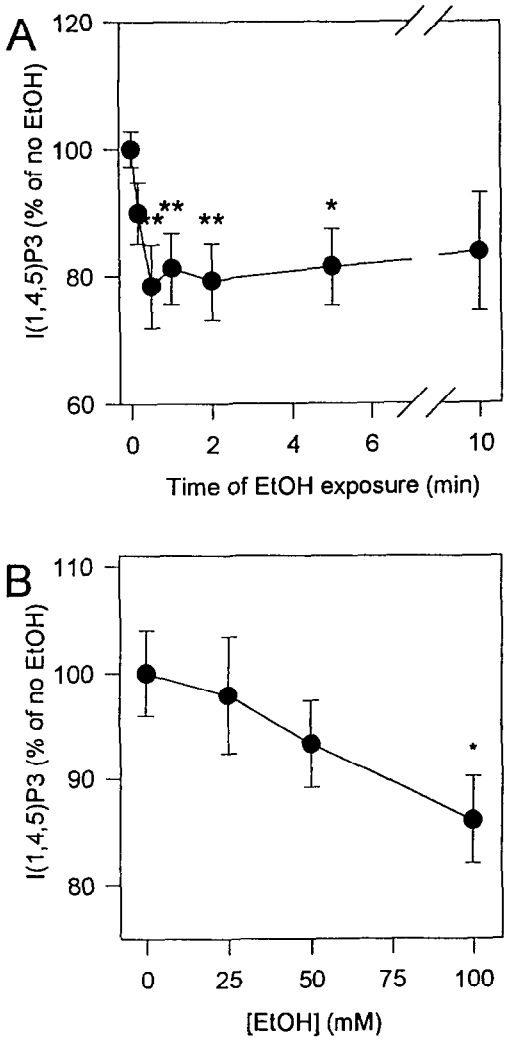

Fig. 3. Time and concentration dependence of ethanol pretreatment on the inhibition of carbachol-induced $I(1,4,5)P_3$ formation in SH-SY5Y cells. (A) 100 mM ethanol was added at indicated times prior to stimulation for 10 sec with 1 mM carbachol. (B) Different concentrations of ethanol were added 5 min prior to stimulation with 1 mM carbachol for 10 sec. Data are expressed as percentage of $I(1,4,5)P_3$ levels in stimulated control cells and are mean ± SEM from three experiments performed in triplicate.* $P < 0.05$;**$P < 0.01$ compared to no added ethanol.

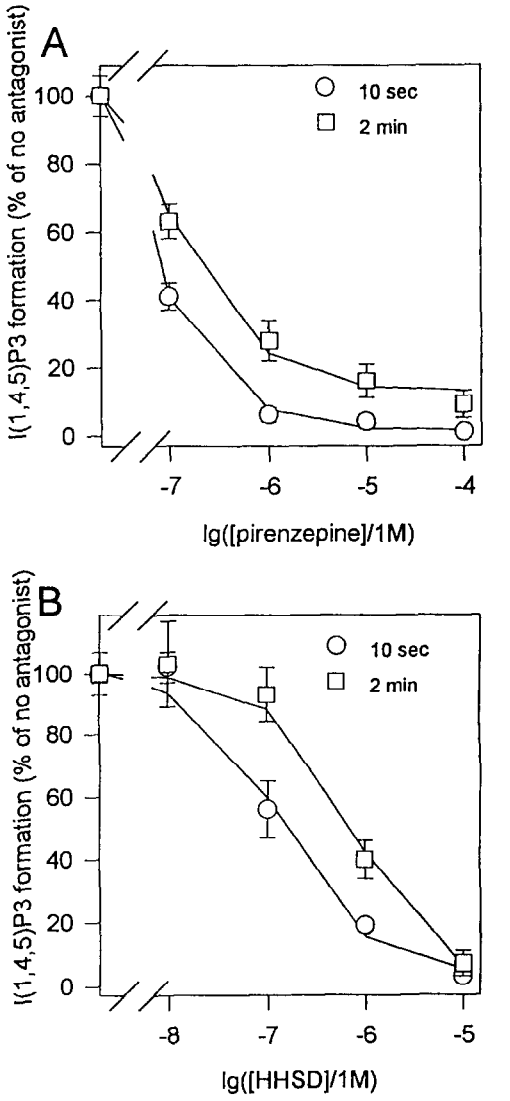

Fig. 4. The effects of the muscarinic antagonists pirenzepine (A) and HHSD (B) on carbachol-induced $I(1,4,5)P_3$ levels after 10 sec (circles) or 2 min (squares) of stimulation. Data are expressed as percentage of $I(1,4,5)P_3$ levels induced in the absence of antagonist and are mean ± SEM from three experiments performed in triplicate.

556 ± 121 fmol/mg protein (mean ± SEM from three separate experiments). The pharmacological constants of the displacement induced by increasing concentrations of PZ and HHSD are demonstrated in Table 2. There was a significant improvement of the fit to the pirenzepine values when a two-site fit was performed, revealing one high-affinity site ($K_{0.5}$ = 23 nM) accounting for 74% of [^{3}H]QNB-binding sites and one low-affinity site ($K_{0.5}$ = 440 nM).

Role of protein kinases and phosphates

To investigate whether the mechanisms of the ethanol effect could involve a cAMP-dependent kinase, protein kinase C, or an okadaic acid-sensitive protein phosphatase, modulators of these enzymes were tested for their ability to influence carbachol-stimulated $I(1,4,5)P_3$ formation (Table 3). Neither the cAMP-dependent protein kinase activator CPTcAMP (2 μM) nor the inhibitor RpcAMPS (100 μM) influenced carbachol-stimulated $I(1,4,5)P_3$ levels, measured after stimulation for 10 sec and 2 min. Addition of the protein kinase C activator PMA (1 μM) significantly diminished $I(1,4,5)P_3$ formation after stimulation with carbachol for 10 sec and 2 min. An inhibitor of protein phosphatases, okadaic acid [11], which has been shown in SH-

Table 2. Inhibition of carbachol-stimulated I(1,4,5)P_3 formation and [^{3}H]QNB-binding to SH-SY5Y cells by pirenzepine and HHSD*

(A) I(1,4,5)P_3 formation	IC_{50} (nM)	K_b (nM)	pK_b
Pirenzepine			
10 sec	64	5.5	8.3 ± 0.1
2 min	162	7.1	8.2 ± 0.1
HHSD			
10 sec	176	15	7.8 ± 0.2
2 min	699	30	7.5 ± 0.1
(B) [^{3}H]QNB binding	$K_{0.5}$	p$K_{0.5}$	% of sites
Pirenzepine (1 site)	64 nM	7.2 ± 0.1	100
Pirenzepine (2 sites, high)	23 nM	7.6 ± 0.1	74 ± 1
Pirenzepine (2 sites, low)	440 nM	6.4 ± 0.1	26 ± 1
HHSD	44 nM	7.3 ± 0.01	100

* Values are calculated as described in the *Materials and Methods* section and are the geometric means (IC_{50}, K_b and $K_{0.5}$) or the algebraic mean ± SEM (pK_b, p$K_{0.5}$ and the % of sites) from three separate experiments.

SY5Y cells to both inhibit protein phosphatase activity [12] as well as potentiate carbachol-stimulated *c-fos* expression [13], did not influence carbachol-stimulated I(1,4,5)P_3 formation.

The effect of PMA suggests that activation of protein kinase C inhibits the carbachol-stimulated formation of I(1,4,5)P_3 in SH-SY5Y cells. In order to elucidate whether ethanol inhibition is mediated via protein kinase C, the ethanol effect was investigated in cells treated with the protein kinase C inhibitors H7, calphostin C [14] and staurosporine prior to stimulation with 100 μM carbachol (Table 4). No significant inhibition by ethanol of carbachol-stimulated I(1,4,5)P_3 levels was apparent in the presence of either H7 or staurosporine and in calphostin C-treated cells ethanol caused a small enhancement of the carbachol-stimulated I(1,4,5)P_3 levels. In unstimulated cells, H7 and calphostin C themselves caused a small increase in I(1,4,5)P_3 levels (21 ± 5 pmol/mg protein in H7-treated cells and 36 ± 5 pmol/mg protein in calphostin C-treated cells compared to levels in control cells of 8 ± 3 pmol/mg protein, N = 6). In addition, there was a significant inhibition by H7 and a significant potentiation by calphostin C of carbachol-stimulated I(1,4,5)P_3 levels in the absence of ethanol.

DISCUSSION

The results from this study demonstrate that acute exposure to ethanol decreases the peak levels of I(1,4,5)P_3 obtained after 10 sec of stimulation with muscarinic agonist whereas steady-state elevation of I(1,4,5)P_3 after 2 min stimulation is unaffected. Previous studies demonstrated that ethanol had differential effects on receptor-stimulated activation of phospholipase C in cell systems from the nervous system. The receptor-stimulated activation of phospholipase C in brain slices was inhibited by acute exposure to ethanol [1, 2], but relatively high

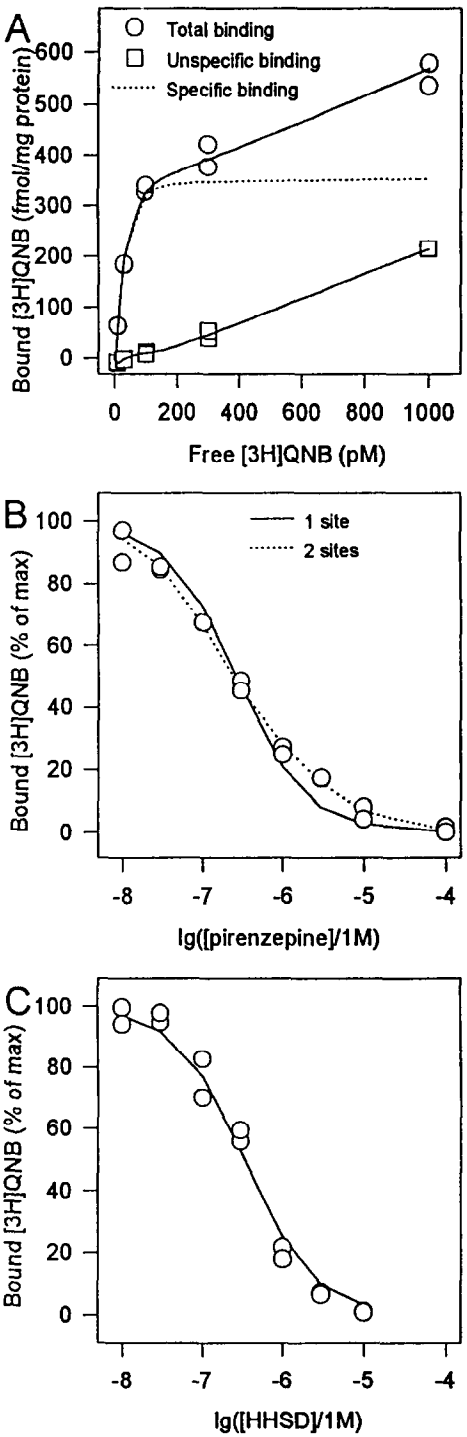

Fig. 5. Binding of [^{3}H]QNB to SH-SY5Y cells and the displacement of this binding by pirenzepine and HHSD. (A) SH-SY5Y cells were incubated with increasing concentrations of [^{3}H]QNB. Total binding, non-specific binding (binding in the presence of 10 μM atropine) and calculated specific binding are demonstrated. (B) and (C) SH-SY5Y cells were incubated with 200 pM [^{3}H]QNB and increasing concentrations of pirenzepine (B) and HHSD (C). The solid continuous lines represent calculated values for a one-site situation and the dashed line in (B) represent a two-site fit, which significantly improved the fit ($P < 0.001$ with the F-test). The data are from one out of three experiments with similar results.

Table 3. The role of protein phosphorylation on carbachol-stimulated $I(1,4,5)P_3$ levels in SH-SY5Y cells*

Addition	$I(1,4,5)P_3$ (% of control stimulation) 10 sec	2 min
CPTcAMP (2 μM)	97 ± 9	111 ± 5
RpcAMPS (100 μM)	92 ± 11	109 ± 7
PMA (1 μM)	30 ± 7†	30 ± 7[a]
OKA (1 μM)	105 ± 5	109 ± 6

* Results are expressed as percent $I(1,4,5)P_3$ of levels in stimulated control cells and are mean ± SEM of six determinations from two separate experiments (CPTcAMPS, RpcAMPS, OKA) and of nine determinations from three separate experiments (PMA).

† $P < 0.001$ compared to stimulated control cells using the *t*-test.

Table 4. The effect of different PKC inhibitors on the ethanol-induced inhibition of carbachol-stimulated $I(1,4,5)P_3$ levels in SH-SY5Y cells*

PKC inhibitor	$I(1,4,5)P_3$ (% of control stimulation) −EtOH	+EtOH
No inhibitor	100 ± 6	76 ± 6[a]
H7 100 μM	82 ± 3[b]	84 ± 12
Calphostin C 3 μM	121 ± 5[c]	142 ± 8[d]
Staurosporine 1 μM	97 ± 8	85 ± 8

* SH-SY5Y cells were incubated in HEPES buffered medium for 15 min prior to stimulation. Five minutes prior to the addition of 100 μM carbachol, 100 mM ethanol and different PKC inhibitors were added to the cells. The cells were stimulated for 10 sec. The data are expressed as percentage of $I(1,4,5)P_3$ levels in stimulated control cells and as mean ± SEM of 8–9 determinations from three separate experiments.

†,‡,§ Significantly different from control stimulation ($P < 0.05$).

¶ Significantly different from stimulated levels without calphostin C ($P < 0.001$) and from cells not exposed to ethanol ($P < 0.05$) using the *t*-test.

ethanol concentrations were needed to obtain this effect. Later studies indicated that the muscarinic receptor-stimulated breakdown of phosphoinositides was particularly ethanol-sensitive during the time of the brain growth spurt, which coincides with maximal density of muscarinic receptors [15, 16]. Studies on cultured cells revealed differing effects of ethanol depending on cell and receptor type. The serotonin-induced formation of inositol phosphates in cultured astrocytes was potentiated by ethanol, whereas stimulation with other agonists was not affected [3]. The formation of inositol phosphates induced by bradykinin in neuroblastoma N1E-115 cells was inhibited by ethanol [5] whereas the same action of bradykinin in neuroblastoma × glioma NG108–15 cells was insensitive to ethanol [4]. Based on the published results it appears likely that ethanol does not exert a general inhibitory effect on the receptor-stimulated formation of $I(1,4,5)P_3$ but rather causes differential effects depending on receptor and/or cell type. However, all these studies were performed using tissue or cells labeled with [^{3}H]-inositol measuring the accumulation of [^{3}H]-inositol phosphates, usually in the presence of Li^+. Thus, it is unlikely that an ethanol effect on specific phases of receptor-stimulated phospholipase C activity would be detected as is the case in the present investigation. The results from this study demonstrate that the ethanol effect on phospholipase C not only varies between receptors and cell types but also with the temporal phase of phospholipase C activity.

Stimulation of receptors coupled to phospholipase C often causes a rapid and transient increase in $I(1,4,5)P_3$ levels, peaking within seconds after the addition of agonist and returning to basal levels within minutes. Such is the case for, for example, bradykinin-induced $I(1,4,5)P_3$ formation in neuroblastoma-glioma NG108–15 cells [17, 18] and carbachol-stimulated formation in neuroblastoma N1E-115 cells [19]. In other cell systems such as astrocytoma 1321N1 cells [20], agonist-stimulated $I(1,4,5)P_3$ levels are not transient but reach a plateau level which is sustained as long as the agonist is present. Furthermore, in some cell types, including striatal neurons [21] and neuroblastoma SH-SY5Y cells [6] and this study, stimulation with carbachol elicits a rapid and transient peak in $I(1,4,5)P_3$ levels declining to a plateau on where it remains for several minutes. One explanation for this response pattern, proposed by Nahorski and co-workers [22], envisions a rapid but partial desensitization of the receptor or other components of the transduction system, resulting in a partial suppression of the early peak level of $I(1,4,5)P_3$ to reach a lower steady-state level in the presence of agonist. Alternatively, this response pattern may be due to a combination of two different mechanisms, one eliciting a rapid and transient peak and a second, inducing a sustained plateau phase of $I(1,4,5)P_3$ levels. In a desensitization model, the ethanol effect on the peak level observed in this study could be explained by an enhancement of the desensitization process. However, the shift to the plateau phase primarily involves a decreased maximal response of the agonist and not a decreased potency of carbachol, as was the case for the ethanol effect. This observation makes it more likely that ethanol affects a component which is selectively involved in the control of the peak level of $I(1,4,5)P_3$. Furthermore, there is evidence that other conditions selectively affect the sustained phase. For instance, in SH-SY5Y cells, the plateau phase appears to be more dependent on Ca^{2+} influx than the peak [22]; in adrenal glomerulosa cells, the myosin light chain kinase inhibitor wortmannin was shown to selectively inhibit the angiotensin II-stimulated sustained increase in $I(1,4,5)P_3$ levels, whereas the initial spikes of $I(1,4,5)P_3$ and Ca^{2+} increases were unaffected [23]. Taken together, these findings indicate that the biphasic $I(1,4,5)P_3$ increase often seen after receptor stimulation is not only due to a rapid and partial desensitization of the same transduction system but may involve separate

components in the two phases subject to differential regulation.

A possible explanation for the selective effect of ethanol on the peak of $I(1,4,5)P_3$ formation is that the two phases are mediated via different muscarinic receptor subtypes, with only one of these being sensitive to ethanol. The SH-SY5Y cell-line appears to display a clonal heterogeneity regarding the nature of the predominant muscarinic receptor subtype coupled to phospholipase C. Results have demonstrated the existence of primarily M_1 receptors [24, 25], M_3 receptors [26, 27] or the coexistence of these two subtypes [28]. The data obtained from the displacement experiments indicate that there are two separate binding sites with different $K_{0.5}$ for pirenzepine similar to published results [24]. The potency for the apparent high affinity site, which constitutes 74% of the total [^{3}H]QNB-binding sites, suggests that these sites are primarily M_1 receptors [29]. The short incubation times required for the functional studies and the apparent non-competitiveness of the muscarinic antagonists in studies using such short stimulation times [30] make it more hazardous to draw definite conclusions regarding the receptor type involved in $I(1,4,5)P_3$ formation. However, the potency of PZ for inhibiting $I(1,4,5)P_3$ formation is in accordance with its potency for inhibition of [^{3}H]QNB-binding. Furthermore, the potency of PZ in our study markedly contrasts with results obtained with a clone of the SH-SY5Y cell line in which carbachol-stimulated Ca^{2+} increase is coupled to muscarinic M_3 receptors [31]. In that study, HHSD was between 50-fold and several hundred-fold more potent than PZ in inhibiting carbachol-stimulated Ca^{2+} increase and noradrenaline release. Taken together, these findings indicate that for the cells used in this report, carbachol-stimulated $I(1,4,5)P_3$ formation is mediated via muscarinic M_1 receptors. Thus, if the two phases of $I(1,4,5)P_3$ formation involve separate components, these are likely to be post-receptor branches of the signal transduction pathway.

Several reports indicate that some acute effects of ethanol on signal transduction components depend on protein kinase C activity. This was observed for the inhibition of vasopressin-stimulated $I(1,4,5)P_3$ formation in hepatocytes [32], increased cAMP levels in platelets [33] and, as demonstrated in a recent study, the inhibition of a serotonergic 5-HT_{1c} stimulated chloride current in *Xenopus* oocytes expressing brain mRNA [34]. The effects of PMA in our study demonstrate that activation of protein kinase C diminishes carbachol-stimulated formation of $I(1,4,5)P_3$. No ethanol inhibition could be detected when the cells were stimulated in the presence of the protein kinase C-inhibitors H7 or calphostin C. These results suggest that protein kinase C activity is essential for the ethanol-induced inhibition of carbachol-stimulated $I(1,4,5)P_3$ levels. However, the protein kinase C inhibitors themselves had disparate effects on $I(1,4,5)P_3$ levels. This could be due either to their lack of specificity and thus to an inhibition of other protein kinases, or to differential inhibitory actions towards different protein kinase C isozymes. In fact, the stimulatory effect of ethanol on phosphatidylethanolamine hydrolysis in NIH 3T3 cells seems to be differently modulated by different protein kinase C isozymes [35, 36]. It is not likely that cAMP dependent protein kinase-sensitive mechanisms are involved in the ethanol effect since neither activation nor inhibition of this enzyme influenced carbachol-stimulated $I(1,4,5)P_3$ formation.

In conclusion, the results of this study demonstrate that stimulation of muscarinic receptors activates a biphasic increase in $I(1,4,5)P_3$ in neuroblastoma SH-SY5Y cells where only the initial peak phase is sensitive to acute exposure to ethanol. This effect of ethanol is dependent on protein kinase C activity.

Acknowledgements—The authors are grateful for the excellent technical assistance of Berit Färjh and Monica Mihailescu. Financial support was obtained from The Swedish Medical Research Council (05249), The Swedish Alcohol Research Fund, The Albert Påhlsson, Crafoord, and Tore Nilsson Foundations, the Swedish Society for Medical Research, and the Medical Faculty at Lund University. J.B.H. is recipient of a visiting scientist fellowship from the Swedish Medical Research Council (10286) and is further supported by U.S. Public Health Service grants AA07186, AA07215, and AA08714.

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