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Functional characterisation of α_1-adrenoceptor subtypes mediating noradrenaline-induced inositol phosphate formation in rat thalamus slices

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Abstract

In cross-chopped slices from rat thalamus and in the presence of 10 mM LiCl, noradrenaline stimulated the accumulation of [^{3}H]inositol phosphates with [^{3}H]inositol monophosphates ([^{3}H]IP_1) being the major product detected ($86 \pm 2\%$ of total [^{3}H]inositol phosphates). Noradrenaline-induced [^{3}H]IP_1 accumulation was concentration-dependent and yielded an EC_{50} of 4.6 ± 0.2 μM, maximum effect of $272 \pm 3\%$ of basal formation and Hill coefficient (n_H) of 1.6 ± 0.1. The effect of 100 μM noradrenaline was inhibited by the α_1-adrenoceptor antagonists prazosin, (+)-niguldipine, 5-methylurapidil and WB-4101 (2-(2,6-dimethoxyphenoxyethyl) aminomethyl-1,4-benzodioxane). The inhibition curve for prazosin best fit to a single-site model whereas curves for (+)-niguldipine, 5-methylurapidil and WB-4101 best fit to a two-site model. The putative α_{1D}-adrenoceptor-selective antagonist BMY 7378 (8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione) showed low potency and efficacy to inhibit the response to noradrenaline. Pre-treatment of the slices with chloroethylclonidine (100 μM; 30 min) decreased by $64 \pm 4\%$ the maximum response. Noradrenaline-induced [^{3}H]IP_1 accumulation was significantly reduced by Ca^{2+} removal (by $64 \pm 2\%$) and by the Ca^{2+}-channel blockers Ni^{2+}, Co^{2+} and nimodipine (inhibition of $56 \pm 6\%$, $54 \pm 5\%$ and $41 \pm 5\%$, respectively). Taken together these results indicate that noradrenaline-induced inositol phosphate formation in thalamus slices is mainly mediated by the activation of both α_{1B} and α_{1A} subtypes of α_1-adrenoceptors.

Keywords: Thalamus; α_1-Adrenoceptor; α_{1A}-Adrenoceptor; α_{1B}-Adrenoceptor; α_{1C}-Adrenoceptor; Inositol phosphate

1. Introduction

The thalamus, a nucleus of special relevance for integrating sensorial and motor information (Kelly and Dood, 1991), is innervated by noradrenergic fibres arising from neurones located in the locus coeruleus (Moore and Bloom, 1979). Both electrical stimulation to locus coeruleus and local application of noradrenaline increase the excitability of thalamic neurones (Rogawski and Aghajanian, 1980; Kayama et al., 1982; Kayama, 1985) and further investigation has revealed that these facilitatory effects are due to an α_1-adrenoceptor-mediated reduction of an ionic current identified as a 'leak' K^+ conductance denominated $I_{K\,leak}$ or I_{KL} (McCormick, 1992).

α_1-Adrenoceptors are among those receptors whose activation leads to breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and diacylglycerol (Summers and McMartin, 1993). $Ins(1,4,5)P_3$ mobilises Ca^{2+} ions from intracellular stores, while diacylglycerol activates protein kinase C (Berridge, 1993). Activation of this pathway involves one or more specific G proteins which, when stimulated by agonist-occupied receptors, activate a membrane-bound, PIP_2-specific phospholipase C (Minneman and Esbenshade, 1994). This mechanism would predict no dependence on extracellular Ca^{2+} ions. However, some functional responses to α_1-adrenoceptor activation are highly dependent on influx of extracellular Ca^{2+} via voltage-dependent as well as non-voltage-dependent Ca^{2+} channels (Minneman, 1988; Summers and McMartin, 1993; Minneman and Esbenshade, 1994). Thus, α_1-adrenoceptor activation appears to stimulate PIP_2 breakdown via two different pathways, direct G protein-coupled activation of phosphalipase C, and agonist-mediated Ca^{2+} entry and Ca^{2+} activation of one or more forms of phospholipase C (Han et al., 1987a; Eberhard and Holz, 1988; Baird and Nahorski, 1990).

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Two α_1-adrenoceptor subtypes (α_{1B} and α_{1A}) have been defined pharmacologically. These subtypes can be distinguished by the antagonists (+)-niguldipine, WB-4101 (2-(2,6-dimethoxyphenoxyethyl) aminomethyl-1,4-benzodioxane) and 5-methylurapidil, known to have some selectivity for the α_{1A} over the α_{1B} subtype, as well as by the alkylating agent chloroethylclonidine, to which α_{1B}-adrenoceptors are highly sensitive (Han et al., 1987b; Summers and McMartin, 1993; Minneman and Esbenshade, 1994; Bylund et al., 1994; Hieble et al., 1995; Michel et al., 1995). There is some evidence that G protein-coupled activation of phospholipase C and Ca^{2+} entry are mainly mediated by α_{1B}- and α_{1A}-adrenoceptor subtypes, respectively (Han et al., 1987a), although more recent data indicate that there is not a clear cut-off distinction in signal transduction mechanisms between subtypes (Esbenshade et al., 1994; Michel et al., 1995).

Evidence for a third subtype of α_1-adrenoceptors has arisen from molecular cloning (Ford et al., 1994; Michel et al., 1995; Hieble et al., 1995). The cloned receptor has been denominated α_{1D} and shows low affinity for (+)-niguldipine and 5-methylurapidil, moderate affinity for WB-4101 and high affinity for the antagonist BMY 7378 (8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione) as well as moderate-high sensitivity to chloroethylclonidine (Michel et al., 1995; Hieble et al., 1995). Little information on tissue distribution and function of the α_{1D} subtype is available so far.

Noradrenaline stimulates PIP_2 breakdown and inositol phosphate formation in slices from rat thalamus (Johnson and Minneman, 1985) where the presence of at least two α_1-adrenoceptors (presumably the α_{1A} and α_{1B} subtypes) has been suggested by the biphasic displacement of ^{125}I-[2-*b*-(4-hydroxy-phenyl)ethylaminomethyl]tetralone (^{125}I-BE) binding by WB-4101, by the reduction in ^{125}I-BE binding after treatment with chloroethylclonidine (Wilson and Minneman, 1989) and by in situ hybridisation studies (McCune et al., 1993). We were unaware of any previous characterisation of functional responses coupled to α_1-adrenoceptor subtypes and in this work we aimed to determine the contribution of such subtypes to inositol phosphate formation in thalamus slices labelled with [^{3}H]inositol by examining the action of α_1-adrenoceptor antagonists with some selectivity on α_{1A}-adrenoceptors (WB-4101, (+)-niguldipine and 5-methylurapidil) as well as that of the putative α_{1D}-adrenoceptor selective antagonist BMY 7378, the action of the alkylating agent chloroethylclonidine and the Ca^{2+} dependence of the response.

2. Materials and methods

2.1. Accumulation of [^{3}H]inositol phosphates

Rats (Wistar strain, males, 250–350 g) were killed by decapitation, the brain was rapidly removed from the skull and thalami from both hemispheres were dissected out. Cross-chopped slices (250×250 μm, McIlwain tissue chopper) were washed three times and incubated at 37°C for 30 min in Krebs-Henseleit (KH) medium (composition in mM: NaCl, 116; KCl 4.7, $MgSO_4$, 1; KH_2PO_4, 1.2; $NaHCO_3$, 25; $CaCl_2$, 2; and D-glucose, 11; pH 7.4) with two further changes of medium. The medium was bubbled continuously with O_2/CO_2 (95:5% v/v). At the end of the equilibration period the slices were washed once more and transferred to a flat-bottom vial and allowed to settle under gravity. Portions of the slices (25 μl, 0.68 ± 0.03 mg protein (Lowry et al., 1951), $n = 10$) were added to 210 μl of KH medium, containing 0.12 μM *myo*-[2-^{3}H]inositol (0.5 μCi per incubation) and 12 mM LiCl (to yield 10 mM as final concentration) in flat-bottom vials. Antagonists, where present, were added at this stage in a 5 μl volume.

The mixture was incubated for 30 min in a shaking water bath before the addition of agonists (10 μl) or 10 μl ascorbic acid (100 μM final concentration) such that the total volume was always 250 μl. The vials were gassed with O_2/CO_2 and, after a further incubation for 60 min (unless otherwise stated), the reaction was stopped by the addition of 250 μl of ice-cold 10% perchloric acid containing 1 mM EGTA and 1 mg · ml^{-1} phytic acid. The time of incubation chosen was that corresponding to the limit of linearity for [^{3}H]inositol monophosphates ([^{3}H]IP_1) accumulation from the time-course (see Section 3). At this time and in the presence of lithium ions, [^{3}H]inositol 1-phosphate, derived from [^{3}H]inositol 1,4,5-trisphosphate, is the major product formed in response to α_1-adrenoceptor activation as determined by HPLC separation (Wigginton and Minneman, 1991).

The samples were left on ice for 30 min and inositol phosphates extracted by the trioctylamine-freon method (Sharpes and McCarl, 1982; Downes et al., 1986). Trioctylamine/1,1,2-trichlorotrifluoroethane (1:1, v/v) (0.4 ml) was added to the incubation mixture, the solution vortex mixed, and the resulting three phases separated by centrifugation at $900 \times g$ for 5 min. A portion (0.35 ml) of the upper, aqueous, phase was mixed with 3 ml of 50 mM Tris buffer, pH 7.4, and the mixture applied to chromatography columns (Bio-Rad Poly-Prep) containing 2 ml of a 1:1 slurry of Dowex AG 1-X8 (formate form, 100–200 mesh; Bio-Rad)/H_2O. [^{3}H]Inositol and [^{3}H]glycerophosphoinositol were removed with 10 ml distilled H_2O and 10 ml 60 mM ammonium formate/5 mM sodium tetraborate, respectively. [^{3}H]Inositol monophosphates ([^{3}H]IP_1) were then eluted into scintillation vials with 10 ml of 200 mM ammonium formate/100 mM formic acid. In some experiments higher phosphates were eluted by sequential addition of 10 ml of 400 mM ammonium formate/100 mM formic acid (for [^{3}H]inositol bisphosphates, [^{3}H]IP_2) and 10 ml of 800 mM ammonium formate/100 mM formic acid (for [^{3}H]inositol trisphosphates, [^{3}H]IP_3). Scintillation liquid (10 ml) was added to each eluted sample and the

tritium content determined by liquid scintillation counting. Within an experiment 3–4 replicate determinations were made of each incubation condition.

Stock solutions of noradrenaline (25 mM) also contained 2.5 mM ascorbic acid. It has been shown previously with rat cerebral cortical slices that [^{3}H]inositol phosphates accumulation induced by noradrenaline is not altered in the presence of inhibitors of neuronal uptake or monoamine oxidase (Kendall et al., 1985). Prazosin, (+)-niguldipine hydrochloride and nimodipine were dissolved in methanol. WB-4101 hydrochloride was dissolved in ethanol. At their final concentrations (2%, v/v) neither methanol nor ethanol affected basal or noradrenaline-induced [^{3}H]IP_1 accumulation (data not shown).

2.2. Pre-treatment of slices with chloroethylclonidine

Following the initial incubation in KH medium for 30 min, thalamus slices were divided in two portions, and one portion treated in 50 ml KH buffer with 100 μM chloroethylclonidine for 30 min at 37°C, with continuous gassing with O_2/CO_2 (95:5, v/v). At the end of this period the slices were thoroughly washed with KH solution and then labelled with [^{3}H]inositol and incubated with noradrenaline as above.

2.3. Ca^{2+} dependence of [^{3}H]IP_1 accumulation

Since varying the extracellular concentration of Ca^{2+} ions alters [^{3}H]inositol labelling of brain tissues (Alexander et al., 1990 and Arias-Montaño and Young, unpublished results) thalamus slices were pre-incubated in normal KH medium, washed thoroughly in buffer with no added Ca^{2+} and then labelled with [^{3}H]inositol in Ca^{2+}-free medium for 30 min. Following this period Ca^{2+} concentrations were adjusted as required with $CaCl_2$ before exposing the slices to noradrenaline.

2.4. Analysis of data

Concentration-response data for both noradrenaline-induced [^{3}H]IP_1 accumulation and antagonist inhibition were fitted by non-linear regression to a four-parameter logistic equation using the program Prism (Graph Pad Software, San Diego, CA, USA). All data are expressed as means ± S.E.M.

For inhibition experiments K_i values were calculated by the program from the IC_{50} estimates according to the Cheng-Prusoff equation (Cheng and Prusoff, 1973; Craig, 1993):

$$K_i = IC_{50}/1 + (A/EC_{50})$$

where A is the concentration of noradrenaline present in the assay (100 μM) and EC_{50} is the concentration of noradrenaline (4.2 μM) required for half-maximal stimulation of [^{3}H]IP_1 accumulation (Fig. 2). Best fit to one-site or two-site competition models was evaluated by the program by using the F-test ($P < 0.05$).

2.5. Drugs

myo-[^{3}H]Inositol (17.2 Ci mmol^{-1}) was purchased from Amersham International. Chloroethylclonidine hydrochloride, prazosin hydrochloride, $S(+)$-niguldipine hydrochloride, (−)-propranolol hydrochloride, WB-4101 (2-(2,6-dimethoxyphenoxyethyl) aminomethyl-1,4-benzodioxane hydrochloride), 5-methylurapidil, BMY 7378 dihydrochloride, methoxamine and nimodipine were obtained from RBI (Natick, MA, USA). Noradrenaline bitartrate, lithium chloride, phytic acid, trioctylamine, 1,1,2-trichlorotrifluoroethane, 2-amino-2-hydroxymethyl-propan-1,3-diol (Tris) were purchased from Sigma (St. Louis, MO, USA). Phenylephrine was kindly provided by Dr. Cristina Paredes (Medical School, UNAM, Mexico).

3. Results

3.1. Characterisation of noradrenaline-induced [^{3}H]inositol phosphates accumulation

The time-course of [^{3}H]IP_1, [^{3}H]IP_2 and [^{3}H]IP_3 accumulation induced by 100 μM noradrenaline in the presence of 10 mM LiCl is shown in Fig. 1. [^{3}H]IP_1 increased linearly with time up to 60 min where the rate of accumulation started to decline. [^{3}H]IP_2 increased linearly up to 40 min when it reached a maximum. Accumulation of [^{3}H]IP_3 was evident after 3 min of incubation but there were no significant changes afterwards. Basal levels of [^{3}H]IP_1, but not [^{3}H]IP_2 and [^{3}H]IP_3, increased slightly, but significantly ($P < 0.05$, Student-Newman-Keuls multiple range test), in a linear manner over the 90 min period. At 60 min [^{3}H]IP_1 accounted for 86 ± 2%, [^{3}H]IP_2 for 13 ± 3% and [^{3}H]IP_3 for 0.9 ± 0.2% of total [^{3}H]inositol phosphates ($n = 3$). Therefore in all subsequent experiments the incubation time with noradrenaline was set at 60 min, only the [^{3}H]IP_1 fraction was collected and all results are referred to this fraction.

[^{3}H]IP_1 accumulation stimulated by noradrenaline was concentration dependent (Fig. 2). Best-fit values for the concentration-response curve yielded an EC_{50} of 4.6 ± 0.2 μM, maximum effect of 272 ± 3% of basal formation and Hill coefficient (n_H) of 1.6 ± 0.1 (best-fit values for the combined data from 3 experiments).

In another series of experiments ($n = 4$), the response to 100 μM noradrenaline (223 ± 6% of basal) was fully blocked by the α_1-adrenoceptor antagonist prazosin (1 μM) (Table 1). The partial α_1-adrenoceptor agonists, phenylephrine and methoxamine (100 μM) also stimulated [^{3}H]IP_1 formation to 165 ± 8 and 171 ± 9% of basal, respectively (53 ± 7% and 58 ± 8% of the response to 100 μM noradrenaline, basal subtracted). The selective β-

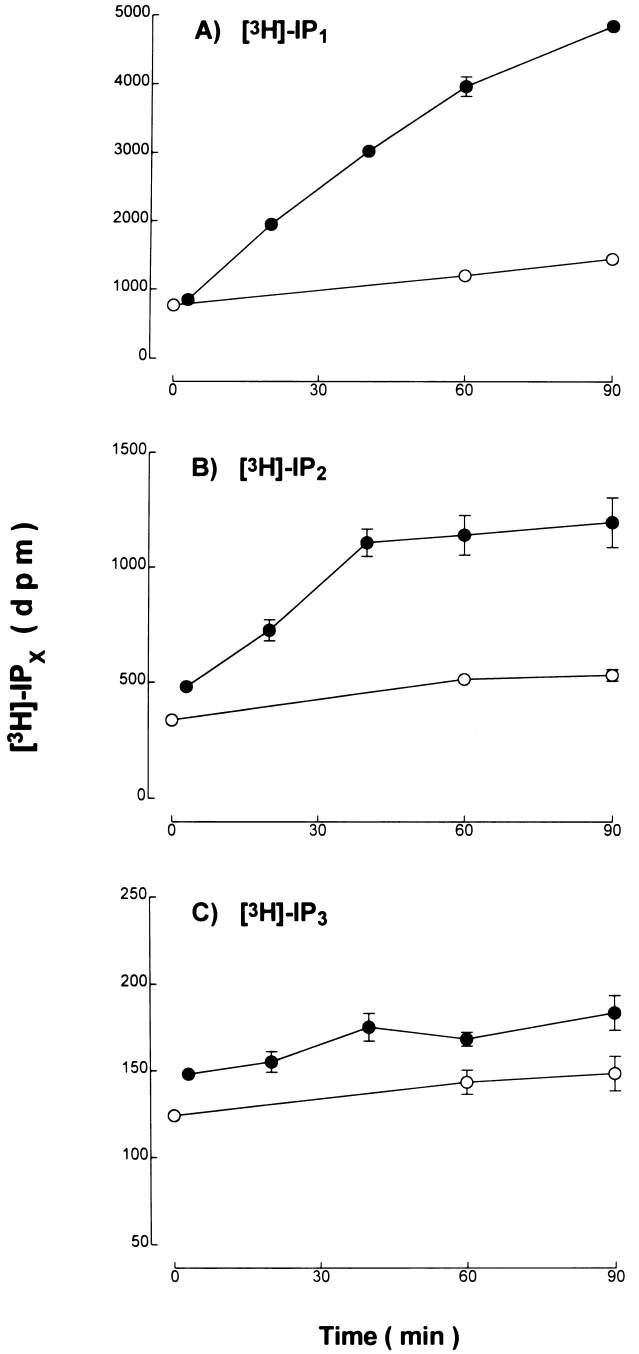

Fig. 1. Time-course of accumulation of [^{3}H]inositol phosphates in the presence and absence of 100 μM noradrenaline. Values are the means ± S.E.M. from triplicate determinations from a single experiment. Where no error bars are shown the error was within the size of the symbol. The whole experiment was repeated three times with similar results. (A) [^{3}H]IP$_1$, (B) [^{3}H]IP$_2$, (C) [^{3}H]IP$_3$. (○) No addition (basal); (●) 100 μM noradrenaline.

adrenoceptor antagonist (−)-propranolol (10 μM) (Summers and McMartin, 1993) did not affect the response to noradrenaline (102 ± 11% of control). Noradrenaline-induced [^{3}H]IP$_1$ formation was slightly, but significantly, reduced by the α_2-adrenoceptor antagonist idazoxan (1 μM), 18 ± 6% inhibition (ANOVA and Dunnett's post-hoc test, Table 1).

Table 1
Effect of adrenergic agonists and antagonists on [^{3}H]IP$_1$ accumulation

	[^{3}H]IP$_1$ accumulation (% basal)	% Response to NA
Basal	100 ± 2	–
NA (100 μM)	223 ± 6	100 ± 5
Methoxamine (100 μM)	171 ± 9	58 ± 8
Phenylephrine (100 μM)	165 ± 8	53 ± 7
Isoproterenol (100 μM)	97 ± 3	0
NA + prazosin (1 μM)	100 ± 4 [a]	0
NA + idazoxan (1 μM)	200 ± 4 [a]	82 ± 6
NA + (−)propranolol (10 μM)	225 ± 12	102 ± 11

Slices were labelled with [^{3}H]inositol and exposed to agonists or to noradrenaline (NA) in the presence or absence of selective antagonists. Values for [^{3}H]IP$_1$ accumulation are the means ± S.E.M. from the combined data from three experiments. Basal accumulation (no additions) was 1766 ± 24 dpm. [a] Values statistically different from noradrenaline alone (ANOVA and Dunnett's post-hoc test, $P < 0.05$).

3.2. Sensitivity to chloroethylclonidine of noradrenaline-induced [^{3}H]IP$_1$ accumulation

Pre-treatment of thalamus slices with 100 μM chloroethylclonidine for 30 min produced a marked depression in the maximum accumulation of noradrenaline-induced [^{3}H]IP$_1$ accumulation (Fig. 3). The maximum response after chloroethylclonidine was 36 ± 4% of that in the untreated slices (basal subtracted). Both the EC_{50} (4.0 ± 0.3 and 8.8 ± 0.9 μM) and the Hill coefficient

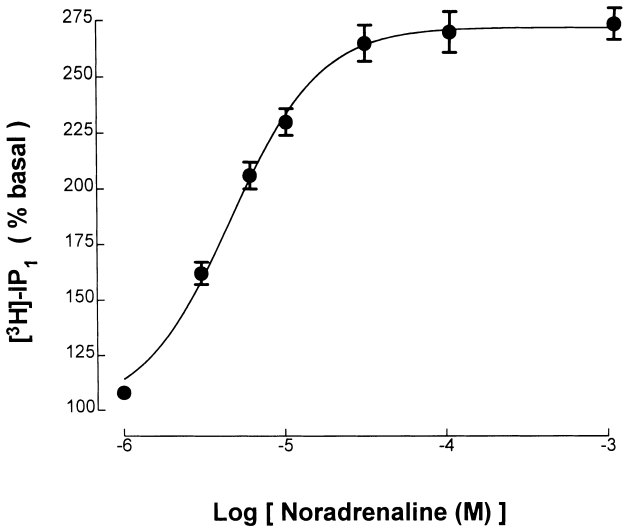

Fig. 2. Concentration-response curve for noradrenaline-induced [^{3}H]inositol monophosphate ([^{3}H]IP$_1$) accumulation. Slices were incubated for 60 min with increasing concentrations of noradrenaline. [^{3}H]IP$_1$ formation is expressed as a percentage of basal accumulation and values are the means ± S.E.M. from the combined data from three experiments. The line drawn is the best fit to a four-parameter logistic equation (see Section 2). Best-fit estimates are given in the text.

(1.5 ± 0.1 and 1.0 ± 0.1) also changed significantly after pre-treatment with chloroethylclonidine ($P < 0.05$, *F*-test), although the much lower response in chloroethylclonidine-treated slices makes the estimates for the best fit less accurate.

3.3. Effect of α_1-adrenoceptor antagonists

The α_1-adrenoceptor antagonists tested (prazosin, (+)-niguldipine, WB-4101 and 5-methylurapidil) reversed in a concentration-dependent manner the [^{3}H]IP_1 accumulation induced by 100 μM noradrenaline (Fig. 4). However, whereas the inhibition curve for prazosin best fit to a single-site model (pK_i 9.3, n_H 0.9 ± 0.2) by non-linear regression analysis (Fig. 4A and Table 2), the curves for (+)-niguldipine, WB-4101 and 5-methylurapidil inhibition best fit to a two-site model (Fig. 4B, C and D and Table 2).

Estimates of pK_i values (Table 2) were for (+)-niguldipine (n_H 0.61 ± 0.06) 10.0 for the high-affinity site (accounting for 57 ± 7% of the response) and 8.3 for the low-affinity site; for WB-4101 (n_H 0.59 ± 0.09) 10.3 for the high-affinity site (accounting for 51 ± 10% of the response) and 8.5 for the low-affinity site; for 5-methylurapidil (n_H 0.58 ± 0.06) 9.7 for the high-affinity site (accounting for 40 ± 8% of the response) and 8.0 for the low-affinity site (see Table 2). The differences in affinity between the high- and low-affinity sites ($K_{i\,high}/K_{i\,low}$) were 50-fold for (+)-niguldipine, 63-fold for WB-4101 and 50-fold for 5-methylurapidil.

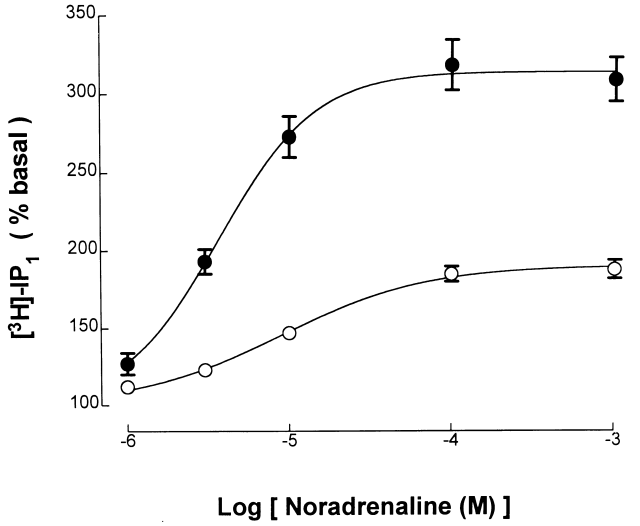

Fig. 3. Effect of chloroethylclonidine on noradrenaline-induced [^{3}H]inositol monophosphate ([^{3}H]IP_1) accumulation. Slices were pre-treated with 100 μM chloroethylclonidine for 30 min and then exposed to 100 μM noradrenaline for 60 min. Values are expressed as a percentage of basal accumulation and correspond to the means ± S.E.M. from the combined data from three experiments. The curves drawn are the best-fit lines to a four-parameter logistic equation (see Section 2). Best-fit estimates are given in the text. The mean basal accumulations were 1485 ± 55 dpm for controls and 1404 ± 31 dpm for chloroethylclonidine-treated slices. (●) Control slices; (○) chloroethylclonidine-treated slices.

Table 2
Inhibition constants of α_1-adrenoceptor antagonists

	n_H	$pK_{i\,high}$	$pK_{i\,low}$	R_{high} (%)
Prazosin	0.90 ± 0.20	9.3 (9.1–9.5)	–	100
(+)-Niguldipine	0.61 ± 0.06	10.0 (9.7–10.4)	8.3 (7.9–8.8)	57 ± 7
WB-4101	0.59 ± 0.09	10.3 (9.4–11.0)	8.5 (7.7–9.4)	51 ± 10
5-Methylurapidil	0.58 ± 0.06	9.7 (9.2–10.4)	8.0 (7.6–8.4)	40 ± 8
BMY 7378	0.80 ± 0.20	6.9 (6.5–7.2)	–	100

Values are the estimates from the best-fit curves for α_1-adrenoceptor-selective antagonists inhibition of noradrenaline (100 μM)-induced [^{3}H]IP_1 accumulation (see Section 2 and Fig. 4). Values within parentheses are 95% confidence intervals.

Fig. 4 also shows that the putative α_{1D}-adrenoceptor-selective antagonist BMY 7378 (E) inhibited noradrenaline-induced [^{3}H]IP_1 accumulation with low efficacy (inhibition of 49 ± 4% at 10 μM, the highest concentration tested) and low potency (pK_i 7.0, n_H 0.8 ± 0.2).

3.4. Ca^{2+} dependence of [^{3}H]IP_1 accumulation

The pattern of Ca^{2+} dependence of [^{3}H]IP_1 accumulation in response to 100 μM noradrenaline is shown in Fig. 5. Ca^{2+} removal significantly reduced noradrenaline-induced [^{3}H]IP_1 accumulation to 36 ± 2% of controls (2 mM Ca^{2+}, $n = 3$). [^{3}H]IP_1 accumulation increased as the Ca^{2+} concentration was raised from nominally zero (no added Ca^{2+}) to 0.25 mM followed by further statistically significant ($P < 0.05$, Student-Newman-Keuls multiple range test) increases as Ca^{2+} was raised from 0.25 to 0.5 mM and from 0.5 to 0.75 mM. Less pronounced, non-significant increases in [^{3}H]IP_1 accumulation were observed when Ca^{2+} concentration was raised from 0.75 to 1.0 mM and from 1.0 to 2.0 mM. The step increases in extracellular Ca^{2+} concentration had no significant effect on basal [^{3}H]IP_1 accumulation.

In line with Ca^{2+} influx being able to activate phospholipase C and thus PIP_2 hydrolysis, [^{3}H]IP_1 accumulation was also stimulated in a concentration-dependent manner by the ionophore A23187, which allows the entry of Ca^{2+} ions into the intracellular space. Concentrations of 1, 3, 10 and 30 μM A23187 augmented [^{3}H]IP_1 formation to 113 ± 5%, 140 ± 3%, 180 ± 4% and 239 ± 5% of basal accumulation, respectively.

To examine further the effect of extracellular Ca^{2+} on noradrenaline-induced [^{3}H]IP_1 accumulation, thalamus slices were incubated with 100 μM noradrenaline in the presence of either divalent cations (1 mM Co^{2+} or Ni^{2+}) or 1 μM nimodipine, an L-type voltage-dependent Ca^{2+} channel blocker (McCarthy and TanPiengco, 1992). Basal accumulation in the presence of Ni^{2+}, Co^{2+} and nimodipine was 89 ± 7, 87 ± 7 and 99 ± 5 of controls, respec-

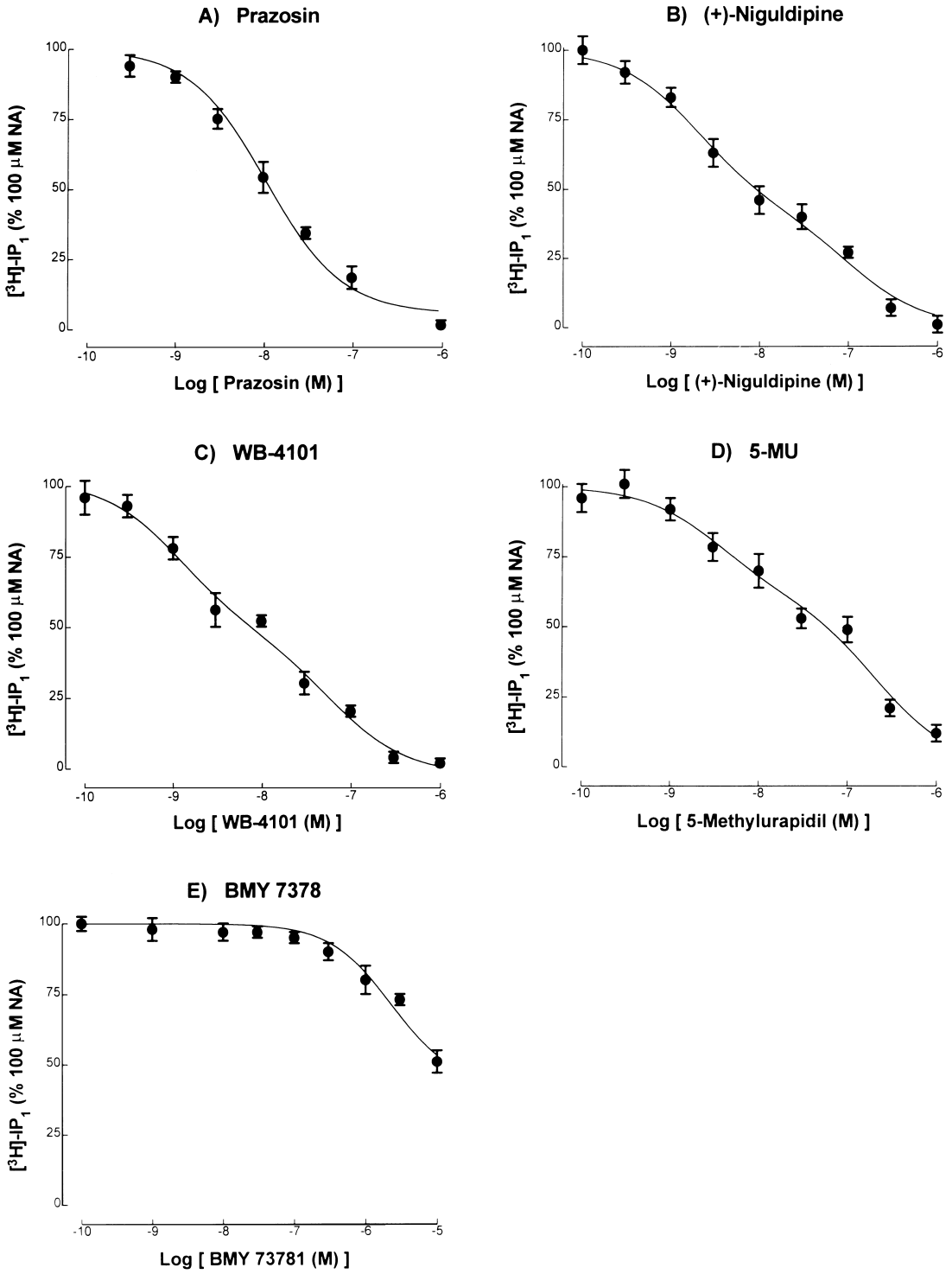

Fig. 4. Inhibition of noradrenaline-induced [^{3}H]inositol monophosphate ([^{3}H]IP$_1$) accumulation by α_1-adrenoceptor antagonists. The indicated concentrations of prazosin (A), (+)-niguldipine (B), WB-4101 (C), 5-methylurapidil, 5-MU (D) and BMY 7378 (E) were examined for [^{3}H]IP$_1$ accumulation in response to 100 μM noradrenaline. Values are expressed as a percentage of the response to noradrenaline in the absence of antagonist and represent the means ± S.E.M. from the combined data from three or four experiments. The curves drawn are the best-fit lines to a four-parameter logistic equation for a one-site model (prazosin and BMY 7378) or a two-site model (WB-4101, (+)-niguldipine and 5-methylurapidil) as described in Section 2. Best-fit estimates are given in the text.

tively (Table 3). None of these values was statistically different from controls (ANOVA and Dunnett's post-hoc test). By contrast Ni^{2+}, Co^{2+} and nimodipine significantly inhibited the response to noradrenaline by 56 ± 6%, 54 ± 5% and 41 ± 5%, respectively ($P < 0.01$, ANOVA and Dunnett's post-hoc test, see Table 3).

Table 3
Effect of Ca^{2+} channel blockers

	Basal	100 μM NA	% Response to NA (basal subtracted)
Control	100 ± 1	258 ± 9	100 ± 5
1 mM Ni^{2+}	89 ± 7	159 ± 3 [a]	44 ± 6
1 mM Co^{2+}	87 ± 7	159 ± 2 [a]	46 ± 5
1 μM nimodipine	99 ± 5	193 ± 4 [a]	59 ± 5

Slices were labelled with [^{3}H]inositol and exposed to noradrenaline (NA) in the presence or absence of Ca^{2+}-channel blockers. Values for [^{3}H]IP_1 accumulation are the weighted means ± S.E.M. from the combined data from three experiments. Basal accumulation (no additions) was 1315 ± 57 dpm. [a] Values statistically different from noradrenaline alone (ANOVA and Dunnett's post-hoc test, $P < 0.01$).

3.5. Effect of WB-4101 in Ca^{2+}-free medium and in chloroethylclonidine-treated slices

In order to test further whether α_{1D}-adrenoceptors were involved in noradrenaline-induced [^{3}H]IP_1 accumulation the effect of WB-4101, an α_1-adrenoceptor antagonist which shows some selectivity for α_{1A}- over α_{1B}-adrenoceptors and for α_{1D}- over α_{1B}-adrenoceptors (Michel et al., 1995), was evaluated in nominally Ca^{2+}-free medium and in slices pre-treated with chloroethylclonidine. Since the sensitivity to chloroethylclonidine of α_{1D}-adrenoceptors is intermediate compared to α_{1A}- and α_{1B}-adrenoceptors (Michel et al., 1995) it is possible that receptors remaining after chloroethylclonidine treatment reflect a mixed α_{1A}- and α_{1D}-adrenoceptor population. As is shown in Fig. 6, in chloroethylclonidine-treated slices the inhibition curve for WB-4101 was biphasic (n_H 0.82 ± 0.07) with pK_i values of 10.4 (95% confidence limits: 10.3–10.6) for the high-affinity site (accounting for 90 ± 3% of the response) and 8.5 (95% confidence limits: 7.6–9.4) for the low-affinity site.

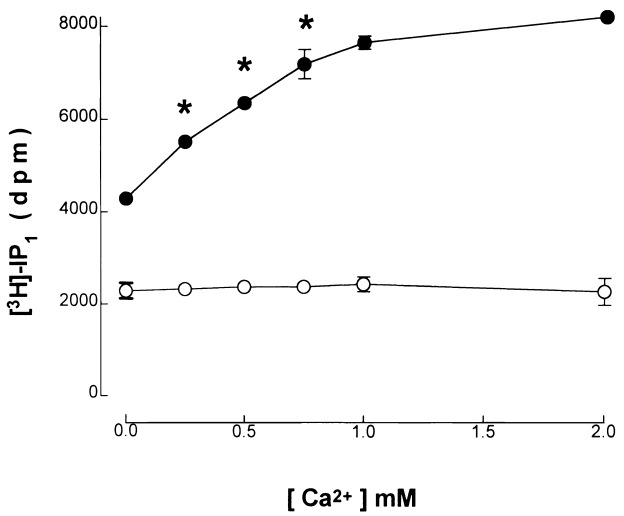

Fig. 5. Ca^{2+} dependence of noradrenaline-induced [^{3}H]inositol monophosphate ([^{3}H]IP_1) accumulation. Values are the means ± S.E.M. from quadruplicate determinations from a single experiment. Where no error bar is shown the error was within the size of the symbol. (○) No addition (basal); (●) 100 μM noradrenaline. The whole experiment was repeated three times with similar results. * $P < 0.05$ with respect to previous value (Student-Newman-Keuls multiple range test).

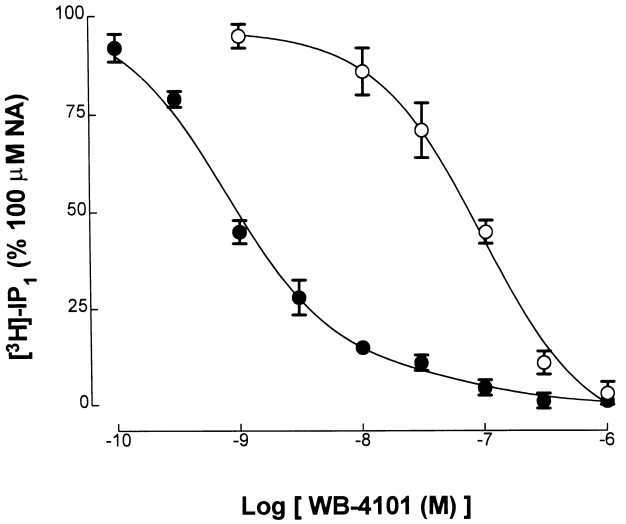

Fig. 6. Inhibition of noradrenaline-induced [^{3}H]inositol monophosphate ([^{3}H]IP_1) accumulation by WB-4101 in chloroethylclonidine-treated slices and in nominally Ca^{2+}-free medium. The indicated concentrations of WB-4101 were examined for [^{3}H]IP_1 accumulation in response to 100 μM noradrenaline in slices treated with chloroethylclonidine (●) as described in Section 2 (100 μM for 30 min) or in slices labelled and incubated in medium with no added Ca^{2+} (○). Values are expressed as a percentage of the response to noradrenaline in the absence of antagonist and represent the means ± S.E.M. from the combined data from three experiments. Absolute values for [^{3}H]IP_1 accumulation were for chloroethylclonidine-treated slices, basal 1134 ± 91 dpm, noradrenaline-stimulated 1858 ± 36 dpm; for slices in nominally Ca^{2+}-free medium, basal 1057 ± 70 dpm, noradrenaline-stimulated 1579 ± 47 dpm. The curves drawn are the best-fit lines to a four-parameter logistic equation for a one-site model (slices in medium with no added Ca^{2+}) or a two-site model (chloroethylclonidine-treated slices) as described in Section 2. Best-fit estimates are given in the text.

In medium with no Ca^{2+} added, where the action of α_{1A}-adrenoceptors on inositol phosphate formation was to be markedly reduced, if not abolished, the inhibition curve for WB-4101 (Fig. 6) was monophasic (n_H 1.1 ± 0.2) with a pK_i value of 8.4 (95% confidence limits: 8.1–8.7).

4. Discussion

Three different subtypes of α_1-adrenoceptors (α_{1A}, α_{1B} and α_{1D}) have been shown to couple to phospholipase C activation and inositol phosphate formation (Minneman and Esbenshade, 1994; Michel et al., 1995). The distinction between subtypes lies mainly on their pharmacology, namely the different affinities of α_1 subtypes for the antagonists WB-4101, 5-methylurapidil, (+)-niguldipine and BMY 7378 and their different sensitivity to chloroethylclonidine ($\alpha_{1B} \geq \alpha_{1D} \gg \alpha_{1A}$). There is also some evidence that G protein-coupled activation of phospholipase C and Ca^{2+} entry (and subsequent activation of Ca^{2+}-sensitive phospholipase C) could be distinctly mediated by

α_{1B}- and α_{1A}-adrenoceptor subtypes, respectively (Han et al., 1987a; Wilson and Minneman, 1990a and reviews by Minneman, 1988; Bylund, 1992; Summers and McMartin, 1993; Minneman and Esbenshade, 1994), although more recent data indicate that there is not a clear-cut distinction between subtypes (Esbenshade et al., 1994; Michel et al., 1995). In contrast with peripheral tissues, which are often enriched in a single subtype (Han et al., 1987b) and with most cell lines, which express exclusively the α_{1B} subtype (Esbenshade et al., 1993), different regions of rat brain, including rat thalamus, contain at least two subtypes (presumably the α_{1A}- and α_{1B}-adrenoceptors), although in different ratios (Wilson and Minneman, 1989). However, little information regarding the proportion in which each subtype contributes to functional responses is available.

In our experiments noradrenaline-induced [^{3}H]IP_1 accumulation in rat thalamus slices can be attributed to α_1-adrenoceptor activation since it was mimicked by two α_1-selective (but partial) agonists, phenylephrine and methoxamine, and was completely blocked by the α_1-adrenoceptor antagonist prazosin ($pK_i > 9$). However, it should be noticed that the response to noradrenaline was slightly but significantly affected ($18 \pm 6\%$ inhibition) by the α_2-adrenoceptor antagonist idaxozan. Thus, although our results indicate that noradrenaline-induced [^{3}H]inositol phosphate formation is fairly mediated by α_1-adrenoceptor activation, some contribution from α_2-adrenoceptors, probably due to potentiation of α_1-mediated responses as proposed by Wilson and Minneman (1991) from observations in primary glial cultures, cannot be ruled out.

Two observations suggest that α_1-adrenoceptor-induced [^{3}H]IP_1 accumulation in thalamus slices corresponds to a mixed response involving more than a single subtype. Firstly, pretreating the slices for 30 min with 100 μM chloroethylclonidine, a concentration high enough to overcome the potential problem of penetration in slices (Minneman, 1988; Kenny et al., 1994), reduced the response to noradrenaline by $64 \pm 6\%$. Whereas this effect strongly suggests that receptors inactivated by chloroethylclonidine (α_{1B} and/or α_{1D}) are involved in such a response, it also indicates that chloroethylclonidine-insensitive receptors participate in noradrenaline-induced [^{3}H]inositol phosphate formation. Further support for at least two distinct subtypes mediating noradrenaline-induced [^{3}H]IP_1 accumulation is given by the inhibition profiles of three α_1-adrenoceptor antagonists, (+)-niguldipine, WB-4101 and 5-methylurapidil, known to have some selectivity (50 to 300-, 20 to 100- and 40 to 80-fold, respectively) for the α_{1A} over the α_{1B} subtype (Gross et al., 1988; Minneman and Esbenshade, 1994). In thalamus slices the inhibition curves for all these three antagonists best fit to a two-site model indicating that at least two receptors with different affinity for such α_1-adrenoceptor antagonists participate in noradrenaline-induced [^{3}H]IP_1 accumulation. In contrast, the inhibition curve for prazosin best fit to a single model ($pK_i = 9.3$) in accordance with previous reports for several tissues (Ford et al., 1994; Minneman and Esbenshade, 1994).

The working definition for the α_{1A}-adrenoceptor is based on its resistance to chloroethylclonidine and high affinity for 5-methylurapidil, WB-4101 and (+)-niguldipine (Ford et al., 1994; Michel et al., 1995). Thus, the high-affinity sites for (+)-niguldipine, WB-4101 and 5-methylurapidil observed in our inhibition curves would correspond to α_{1A}-adrenoceptors (pK_i values of 10.0, 10.3 and 9.7, respectively) whereas the low-affinity sites would then correspond to the α_{1B} subtype (pK_i values of 8.3, 8.6 and 8.0, respectively). These pK_i values, as well as the differences in affinity for the high- and low-affinity sites (63-fold for WB-4101 and 50-fold for (+)-niguldipine and 5-methylurapidil) are similar to those reported for α_{1A} and α_{1B} subtypes in several tissues on the basis of binding and functional studies (Gross et al., 1988; Hanft and Gross, 1989; Ford et al., 1994; Minneman and Esbenshade, 1994; Michel et al., 1995).

The relatively low efficacy (inhibition of 49% of the response at 10 μM) and low potency (pK_i 6.9) of the putative α_{1D}-adrenoceptor-selective antagonist BMY 7378 (pK_i for α_{1D}-adrenoceptors 8.1 ± 0.1; Saussy et al., 1994) to inhibit noradrenaline-induced [^{3}H]IP_1 accumulation in thalamus slices makes the contribution of α_{1D}-adrenoceptors unlikely. Further support for little or no participation of α_{1D}-adrenoceptors in noradrenaline-mediated inositol phosphate formation is given by WB-4101-mediated inhibition in chloroethylclonidine-treated slices (where both α_{1A}- and α_{1D}-adrenoceptors are expected to be functional) and in nominally Ca^{2+}-free medium (where both α_{1B}- and α_{1D}-adrenoceptors would be participating in the response to noradrenaline). In chloroethylclonidine-treated slices the inhibition curve was biphasic but 90% of the response corresponded to the high-affinity site whose pK_i value is that expected for α_{1A}-adrenoceptors whereas the pK_i estimated for the low-affinity site (8.5), responsible of the remaining 10% of the response, is very close to that for the α_{1B} subtype. In nominally Ca^{2+}-free medium the inhibition curve was monophasic with a pK_i value (8.4) that approximates more to that reported for α_{1B}-adrenoceptors (8.4 ± 0.4) than to the value for cloned α_{1D}-adrenoceptors (8.8 ± 0.4; Michel et al., 1995).

There is evidence that α_{1A}-adrenoceptors are associated to Ca^{2+} entry and Ca^{2+} phospholipase C activation (Han et al., 1987a; Eberhard and Holz, 1988; Minneman, 1988; Summers and McMartin, 1993; Minneman and Esbenshade, 1994) and the step increase in noradrenaline-induced [^{3}H]IP_1 formation observed in this study in response to increasing concentrations of Ca^{2+} (from nominally zero Ca^{2+} to 0.75 mM) indicates that Ca^{2+} entry favoured by concentration gradients is playing an active role in noradrenaline-induced PIP_2 hydrolysis in thalamus slices. In line with such a role for Ca^{2+} ions the response to noradrenaline was partially blocked by two non-selective Ca^{2+} channel blockers, Ni^{2+} and Co^{2+} ($56 \pm 6\%$ and

54 ± 5% inhibition, respectively). The use of more potent Ca^{2+} channel blockers such as Cd^{2+} or La^{3+} was prevented by the insolubility of their carbonate salts.

It has been proposed that α_{1A}-mediated Ca^{2+} entry takes place, at least in part, through voltage-operated Ca^{2+} channels (Minneman and Esbenshade, 1994) and the inhibition (41 ± 5%) by nimodipine, a potent blocker of L-type voltage-operated Ca^{2+} channels (McCarthy and Tan-Piengco, 1992) found here indicates that such channels are also involved in Ca^{2+} entry and thus in noradrenaline-induced-[^{3}H]IP_1 accumulation in thalamus slices. The stimulation of [^{3}H]IP_1 accumulation by the Ca^{2+} ionophore A23187 provides further support for Ca^{2+} entry being able to stimulate PIP_2 breakdown and inositol phosphate formation in slices from rat thalamus. Ca^{2+} influx and subsequent inositol phosphate formation mediated by α_{1A}-adrenoceptors has been reported to be pertussis toxin sensitive (Wilson and Minneman, 1990b). Thus, the existence of a functional coupling, via a G protein, between α_{1A}-adrenoceptor activation and the opening of Ca^{2+} channels appears highly likely.

Taken together the pattern of Ca^{2+} dependence and the effect of Ca^{2+} blockers strongly suggest that, at least in part, noradrenaline-induced [^{3}H]IP_1 accumulation is due to Ca^{2+} entry. A similar dependence on extracellular Ca^{2+} of noradrenaline-induced [^{3}H]inositol phosphate formation has been also observed in cerebrocortical slices (Knepper and Rutledge, 1987; Claro et al., 1993) and in primary cultures from whole brain (Wigginton and Minneman, 1991), although no correlation to α_1-adrenoceptor subtypes was made. Likewise in rat whole-brain synaptoneurosomes noradrenaline-induced phosphoinositide hydrolysis has been suggested to involve noradrenaline-stimulated Ca^{2+} influx (Chandler and Crews, 1990). There is evidence that inositol phosphate formation induced by α_1-adrenoceptor-mediated G protein activation and noradrenaline-induced Ca^{2+} influx share physiological characteristics despite the different pathways involved. Thus, in primary glial cell cultures, where the response to the calcium ionophore A23187 and noradrenaline showed a similar dependence on extracellular Ca^{2+}, ionophore-mediated and receptor-mediated responses were not additive (Pearce et al., 1986; Wigginton and Minneman, 1991), suggesting that they are acting on the same pool of phospholipids by activating the same isoform or isoforms of phospholipase C. Furthermore, Wigginton and Minneman (1991) compared by HPLC separation the inositol phosphates formed in response to ionophore and noradrenaline and found that in both conditions inositol 1-phosphate (Ins(1)P) was the predominant inositol phosphate accumulated in the presence of lithium ions, suggesting a similar metabolic pathway.

Although the actual pattern observed for noradrenaline-induced responses in a given tissue would be expected to depend on the relative amounts of α_1 subtypes, the fraction of the response mediated by α_{1A}- and α_{1B}-adrenoceptors estimated from inhibition curves for thalamus slices does not necessarily indicate the relative numbers of α_{1B}- and α_{1A}-adrenoceptors, since it cannot be assumed that there would be a simple relationship between Ca^{2+} entry and phosphoinositide hydrolysis. However, the values for the low-affinity site (presumably the α_{1B} subtype) contribution, as estimated by antagonist inhibition (43 ± 7, 49 ± 10 and 60 ± 8% from (+)-niguldipine, WB-4101- and 5-methylurapidil-mediated inhibition, respectively, see Table 2) are in reasonable agreement with those estimated by Wilson and Minneman (1989) for the low-affinity site in two-site analysis of WB-4101 inhibition of ^{125}I-BE binding (70%) and by measuring ^{125}I-BE binding after chloroethylclonidine pre-treatment (50%).

In summary, our results indicate that noradrenaline-induced inositol phosphate formation in thalamus slices is mediated by activation of more than one subtype of α_1-adrenoceptors. These results also suggest that the functional response involves mainly the α_{1A} and α_{1B} subtypes with little or no evidence for the participation of the α_{1D} subtype. In keeping with α_{1A}-adrenoceptors being involved these data presented herein indicate that Ca^{2+} entry plays an active role in the functional response.

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