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α_{2A}-ADRENOCEPTORS MEDIATE ACTIVATION OF NON-SELECTIVE CATION CHANNELS VIA G_i-PROTEINS IN HUMAN ERYTHROLEUKAEMIA (HEL) CELLS

NO EVIDENCE FOR A FUNCTIONAL ROLE OF IMIDAZOLINE RECEPTORS IN MODULATING CALCIUM

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Abstract—Human erythroleukaemia (HEL) cells were investigated to characterize their α_2-adrenoceptor and imidazoline receptor sites. Membranes from HEL cells bound [^{3}H]2-(2-methoxy-1, 4-benzodioxan-2yl)-2 imidazoline ([^{3}H]RX821002) in a saturable and specific manner with a K_D of 0.64 ± 0.07 nM and a B_{max} of 126 ± 4 fmol/mg protein. [^{3}H]RX821002 was displaced from HEL membranes by adrenergic drugs with the order of potency being yohimbine ≈ oxymetazoline > > prazosin = 2-[2-[4-(*o*-methoxyphenyl)piperazin-1-yl]ethyl]-4,4-dimethyl-1,3(2*H*,4*H*)-isochinolindione HCl (ARC 239), consistent with this site being an α_{2A}-adrenoceptor. HEL membranes also bound [^{3}H]idazoxan in the presence of adrenaline to block α_2-adrenoceptors. This binding was saturable and specific with a K_D of 3.5 ± 1.0 nM and a B_{max} of 31 ± 6 fmol/mg protein. Adrenergic drugs from both the phenylethylamine and imidazoline classes increased high-affinity GTPase activity, an index of activation of regulatory heterotrimeric guanine-nucleotide binding proteins (G-proteins), and produced increases in cytosolic free calcium concentration ($[Ca^{2+}]_i$). The effects of these agonists in both systems were abolished by pertussis toxin pretreatment, and oxymetazoline and clonidine were antagonists. The potency of adrenergic drugs to inhibit 5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline (UK 14304)-induced increases in $[Ca^{2+}]_i$ was yohimbine ≈ oxymetazoline > > ARC 239, consistent with the binding data and an action at α_{2A}-adrenoceptors. No evidence was found for a role of imidazoline receptors in stimulating G-proteins or modulating $[Ca^{2+}]_i$. The adrenergic agonist-induced increases in $[Ca^{2+}]_i$ were due to both release of Ca^{2+} from intracellular stores and entry of extracellular Ca^{2+}. Ca^{2+} entry was blocked by 1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenylethyl}-1*H*-imidazole hydrochloride (SKF 96365), but not by nitrendipine. Adrenaline also stimulated Mn^{2+} entry in HEL cells. Taken together, these results suggest that HEL cells have α_{2A}-adrenoceptors that activate non-selective cation channels via pertussis toxin-sensitive G-proteins, i.e. G_i-proteins.

Key words: HEL cells; α_2-adrenoceptors; imidazoline binding sites; calcium entry; RX821002, SKF 96365

α_2-Adrenoceptors are now recognized as being capable of mediating effects other than the classically described inhibition of adenylate cyclase (for reviews see Refs. 1, 2). For example, stimulation of α_2-adrenoceptors on vascular smooth muscle results in an increase in $[Ca^{2+}]_i$† [3, 4] apparently independent of changes in cyclic AMP concentration. This action of α_2-adrenoceptors is still poorly understood as these effects are difficult to dissect from α_1-adrenoceptor-mediated effects, and as vascular smooth muscle cells are difficult to work with. Also, there are few cell lines with α_2-adrenoceptor-induced increases in $[Ca^{2+}]_i$ to serve as models. HEL cells are a tumour line with megakaryocyte markers [5, 6] that grow in suspension culture and have been used as a model of platelets [7–9]. However, the α_2-adrenoceptor subtype of these cells has not yet been determined. These cells have α_2-adrenoceptor-stimulated increases in $[Ca^{2+}]_i$ [8, 9] and thus may be a suitable model for the vascular smooth muscle α_2-adrenoceptors. HEL cells have also been reported to possess imidazoline binding sites [10]. Imidazoline binding sites bind α-adrenoceptor-selective drugs of the imidazoline and oxazoline classes, but not phenylethylamines [11–13]. Brain stem imidazoline binding sites apparently regulate blood pressure [11–13]. However, there is virtually no information on either the functional significance of these receptors in other tissues or their signal transduction mechanisms.

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† Abbreviations: ARC 239, 2-[2-[4-(*o*-methoxyphenyl) piperazin-1-yl]ethyl]-4,4-dimethyl-1,3(2*H*,4*H*)-isochinolindione HCl; BHT920, 5-allyl-2-amino-5,6,7,8-tetrahydro-4*H*-thiazolo- [4,5-*d*]azepin- dihydrochloride; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; db-cAMP, dibutyryl cyclic AMP; EC_{50}, concentration producing 50% of maximal effect; fura-2/AM, fura-2 acetoxymethylester; G-protein, regulatory heterotrimeric guanine-nucleotide binding proteins; HEL cells, human erythroleukaemia cells; IC_{50}, concentration causing 50% inhibition; NSC channels, non-selective cation channels; SKF 96365, 1-{β-[3-(4-methoxyphenyl)propoxy] - 4 - methoxyphenylethyl} - 1*H* - imidazole hydrochloride; RX821002, 2-(2-methoxy-1,4-benzodioxan-2yl)-2 imidazoline; UK 14304, 5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline.

The aim of this study was to characterize the α_2-adrenoceptors of HEL cells with respect to the specific subtype present, stimulation of G-proteins and increases in $[Ca^{2+}]_i$. We also wished to investigate the imidazoline binding site in greater detail. We report here that HEL cells possess α_{2A}-adrenoceptors which activate NSC channels via pertussis toxin-sensitive G-proteins. HEL cells also possess an imidazoline binding site, but drugs with high affinity for imidazoline binding sites neither stimulate high-affinity GTPase nor alter $[Ca^{2+}]_i$.

MATERIALS AND METHODS

Materials. Adrenaline HCl, isoprenaline HCl, clonidine HCl, oxymetazoline HCl, phentolamine HCl and yohimbine HCl were all obtained from Sigma Chemie (Deisenhofen, Germany). α-Methylnoradrenaline was purchased from Research Biochemicals Incorporated (MA, U.S.A.). ATP and fura-2/AM were purchased from Boehringer Mannheim (Mannheim, Germany). ARC-239, BHT 920, cirazoline, UK 14304 and SKF 96365 were kind gifts from Karl Thomae (Biberach, Germany), Boehringher Ingelheim (Ingelheim, Germany), Synthelabo (Bagneaux, France), Pfizer (Sandwich, U.K.) and Dr J. E. Merritt, SmithKline Beecham (Welwyn, U.K.), respectively. [^{3}H]RX821002 (57 Ci/mmol) and [^{3}H]idazoxan (51 Ci/mmol) were obtained from Amersham (Braunschweig, Germany). Pertussis toxin was obtained from List Biological Laboratories (Campbell, U.S.A.). All other reagents were of analytical grade or the best available commercial grade and obtained from standard commercial suppliers. Compounds were initially dissolved in deionized water, except adrenaline which was dissolved in 1 mM HCl, prazosin which was dissolved in a solution consisting of 0.2 mL glycerol, 3.3 mL 5% (w/v) glucose and 6.5 mL deionized water to give a 1 mM stock and fura-2/AM which was dissolved in DMSO to give a 2 mM stock. Unless otherwise stated, stock agonist and antagonist solutions were of 10 mM and stored at −20°.

Cell culture. HEL cells obtained from the American Type Culture Collection (Rockville, U.S.A.) were grown in suspension culture in RPMI-1640 medium supplemented with 10% (v/v) foetal calf serum, 1% (v/v) non-essential amino acids, 2 mM L-glutamine, 50 U of penicillin/mL and 50 μg of streptomycin/mL in a humidified atmosphere with 7% CO_2 at 37° [9]. Cells were harvested by centrifugation for 10 min at 250 *g*. Cell preparations contained more than 98% viable cells as judged by Trypan Blue dye exclusion.

Cells were pretreated by adding either pertussis toxin (100 ng/mL) or vehicle to the culture medium 24 hr before harvesting the cells. This treatment resulted in ADP-ribosylation of virtually all G_i-protein α-subunits in HEL cells (data not shown).

Preparation of membranes. HEL cells were homogenized by nitrogen cavitation and membranes prepared as described in Seifert and Schultz [14]. Aliquots of cell membranes were thawed and of 10 mM triethanolamine–HCl, pH 7.4. Membranes were suspended at either 0.2–0.4 mg/mL (GTPase assay) or 1.5–2.0 mg/mL (binding assay) in the above buffer and were immediately used for the respective assays. Protein was determined by the method of Lowry *et al.* [15].

Binding. Membranes were thawed and prepared as described above. Total binding was determined by incubation of membranes (50–70 μg of protein/tube) in a buffer containing 0.5 mM $MgCl_2$, 1 mM EDTA and 50 mM Tris–HCl, pH 7.4 (final volume 100 μL). In addition, the reaction mixtures contained the various drugs and hormones at the final concentrations indicated in the Results section. The assay solution was preincubated at 25° for 3 min without radioligand, and binding was initiated by addition of the appropriate radioligand ([^{3}H]-RX821002 or [^{3}H]idazoxan) at the concentrations described below. Non-specific binding was determined in the presence of phentolamine (10 μM) for [^{3}H]RX821002 and the combination of adrenaline (10 μM) and idazoxan (10 μM) for [^{3}H]idazoxan. After 30 min the assay mixtures were filtered through glass fibre filters (Whatman GF/B) presoaked with 1% (w/v) polyethylene imine (Fluka, Buchs, Switzerland). The filters were washed twice with 5 mL of ice-cold buffer consisting of 0.5 mM $MgCl_2$ and 50 mM Tris–HCl, pH 7.4. The radioactivity trapped on the filters was determined by liquid scintillation spectroscopy. For saturation binding the concentration of [^{3}H]RX821002 varied from 0.125 nM to 10 nM and the concentration of [^{3}H]idazoxan varied from 1 nM to 30 nM. For displacement experiments the concentration of [^{3}H]RX821002 was 3 nM. The number of sites (B_{max}) and K_D were determined from non-linear curve fitting of the saturation curve using the program Sigmaplot 4.0 (Jandel, U.S.A.). K_i values (displacement of [^{3}H]RX821002 binding) and K_B values (inhibition of agonist-induced increases in $[Ca^{2+}]_i$ values were calculated using the equation of Cheng and Prusoff [16] from IC_{50} values determined by non-linear curve fitting to the logistic equation.

Measurement of high-affinity GTPase activity. [γ-^{32}P]GTP was synthesized according to the method of Johnson and Walseth [17]. High-affinity GTPase activity was measured according to the method of Seifert *et al.* [18] with minor modifications. The reaction mixtures (100 μL) contained membranes (5.0–7.0 μg protein/tube), 0.5 μM [γ-^{32}P]GTP (0.1 μCi/tube), 5 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM ATP, 1 mM adenosine 5′-[β,γ-imido]-triphosphate, 5 mM creatine phosphate, 40 μg creatine kinase, 1 mM dithiothreitol and 0.2% (w/v) BSA in 50 mM triethanolamine–HCl, pH 7.4. In addition, the reaction mixtures contained the various drugs and hormones at the final concentrations indicated in the Results section. The reaction mixtures were preincubated at 25° for 3 min, then the reaction was started by addition of [γ-^{32}P]GTP. Reactions were terminated after 15 min by the addition of activated charcoal in 20 mM KH_2PO_4, pH 2.0. Low-affinity GTPase activity was determined in the presence of a high concentration of GTP (50 μM) and was subtracted from the total GTPase activity measured at 0.5 μM GTP. Low-affinity

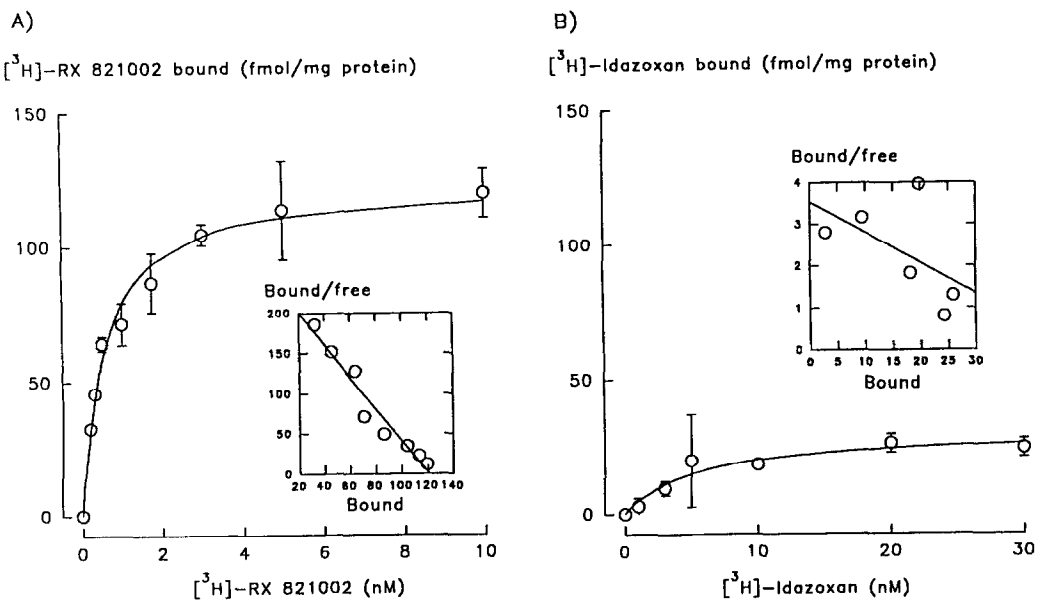

Fig. 1. Saturation experiments for binding of [^{3}H]RX821002 and [^{3}H]idazoxan in HEL membranes. The insets show Scatchard plots of the binding data. A: Membranes were incubated with [^{3}H]RX821002 (0.125–10 nM) as described in Materials and Methods. Non-specific binding was determined with phentolamine (10 μM) and was 19 ± 5% of total binding at saturation. B: Membranes were incubated with [^{3}H]idazoxan (1–30 nM) in the presence of adrenaline (10 μM) as described in Materials and Methods. Non-specific binding was determined with idazoxan (10 μM) and was 73 ± 1% of total binding at saturation. Data are means ± SD of triplicate determinations of an experiment typical of at least three separate experiments.

GTPase activity in HEL membranes was less than 20% of the total GTPase activity (data not shown).

Measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was determined with the dye fura-2/AM as previously described [9, 18]. Briefly, HEL cells were suspended at 1×10^7cells/mL in a buffer consisting of 138 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.1 mM $CaCl_2$, 1 mM Na_2HPO_4, 5 mM $NaHCO_3$, 5.5 mM glucose and 20 mM HEPES, supplemented with 1% (w/v) BSA and adjusted to pH 7.4 with NaOH. Fura-2/AM was added to give a final concentration of 4 μM and cells were incubated for 10 min at 37°. Thereafter, the cells were diluted to 5×10^6cells/mL in the above buffer and incubated for a further 45 min. Following this incubation period the cells were diluted to 0.5×10^6 cells/mL and were centrifuged for 10 min at 250 *g* to remove extracellular fura-2 and unmetabolized fura-2/AM. The cells were then resuspended in the above buffer at 1×10^6cells/mL and kept at room temperature until measurement of $[Ca^{2+}]_i$. One millilitre of this suspension was added to acryl fluorescence cuvettes (Sarstedt, Nümbrecht, Germany) and further diluted with 1 mL of the above buffer. Unless stated otherwise experiments were carried out in the presence of 1 mM extracellular $CaCl_2$. Fluorescence was determined at 37° under constant stirring at 1×10^3 rpm, using a Ratio II spectrofluorometer (Aminco, MD, U.S.A.). Cells were incubated for 3 min prior to addition of stimuli. The excitation wavelength was 340 nm and the emission wavelength was 500 nm. Fluorescent signals were calibrated after lysis of the cells with 0.1% (w/v) Triton X-100 (maximal fluorescence) and subsequent addition of 20 mM EGTA (minimal fluorescence). Peak cytosolic $[Ca^{2+}]_i$ values were calculated according to equation 6 in Grynkiewicz *et al.* [19].

Manganese quenching of the fluorescence of cytosolic fura2 at its isobestic wavelength was used as an index of activation of NSC channels [20]. Manganese quenching experiments were performed on the Ratio II spectrofluorometer with an excitation wavelength of 360 nm and an emission wavelength of 510 nm as described in Ref. 21. Fifty micromolar Mn^{2+} was added to nominally calcium-free buffer 1 min before the stimulus, and the decrease in fluorescence after addition of the stimulus was followed for 2 min. In preliminary experiments this concentration of Mn^{2+} was determined to provide the optimum discrimination between stimulation-induced and non-specific Mn^{2+} entry [20, 21].

RESULTS

Binding

[^{3}H]RX821002 bound to membranes from HEL cells in a specific and saturable manner, with a K_D of 0.64 ± 0.07 nM and a B_{max} of 126 ± 4 fmol/mg protein (Fig. 1). The Scatchard plot was linear, consistent with a single site being present. Adrenergic compounds inhibited the binding of [^{3}H]RX821002

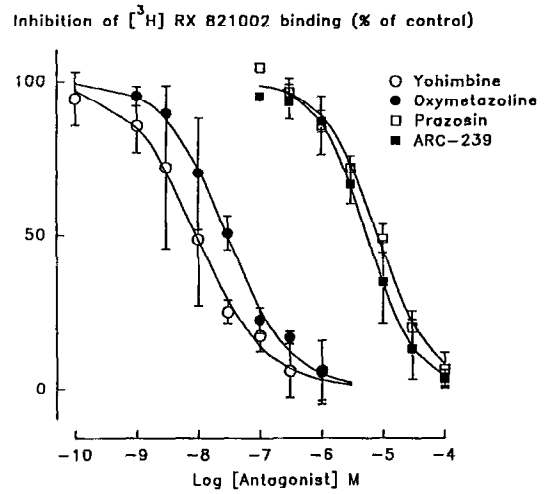

Fig. 2. Inhibition of [^{3}H]RX821002 binding to HEL membranes by adrenergic drugs. Membranes were incubated with 3 nM [^{3}H]RX821002 in the presence of drugs at increasing concentrations as described in Materials and Methods. Data are expressed as percentage of control and represent means ± SD of two to three separate experiments performed in triplicate. When error bars are smaller than the symbol size they are not shown.

with the following order of potency: yohimbine ≈ oxymetazoline > > prazosin = ARC-239 (Fig. 2). The displacement curves were monophasic, also consistent with one binding site. The pK_i values for these antagonists are shown in Table 1 and compared with pK_i values of the above antagonists for α_{2A}-adrenoceptors in human platelets and α_{2B}-, α_{2C}- and α_{2D}-adrenoceptors in various cell types. The pK_i values of the HEL α_2-adrenoceptor correlated more closely with the pK_i values of the α_{2A}-adrenoceptor subtype ($r = 0.98$) than the α_{2D}-subtype ($r = 0.93$), the α_{2C}-subtype ($r = 0.56$) or the α_{2B}-subtype ($r = 0.1$). The ratios of the K_i values of oxymetazoline and yohimbine, and prazosin and yohimbine of the HEL membranes most closely resembled the ratios found for the α_{2A}-adrenoceptor subtype of human platelets (Table 1).

HEL membranes also appeared to have a non-adrenergic binding site, as [^{3}H]idazoxan, in the presence of 10 μM adrenaline to block α_2-adrenoceptors, also bound in a specific and saturable manner with a K_D of 3.5 ± 1.0 nM and a B_{max} of 31 ± 6 fmol/mg protein (see Fig. 1). However, the binding of [^{3}H]idazoxan was too small to permit accurate displacement studies to be performed (see also Brown *et al.* [22]).

High-affinity GTPase activity

Both phenylethylamine and non-phenylethylamine α_2-adrenoceptor agonists produced concentration-dependent increases of high-affinity GTPase activity, an index of G-protein activation [23, 24], in HEL membranes, α-Methylnoradrenaline and UK 14304 were the most effective, followed by BHT 920 and *p*-amino clonidine (Fig. 3). The adrenergic agonist-induced stimulation of high-affinity GTPase was abolished by pretreatment with pertussis toxin (Table 2). Oxymetazoline, clonidine and cirazoline produced no significant activation of high-affinity GTPase. Indeed, oxymetazoline was as potent and effective a blocker of UK 14304-induced stimulation of high-affinity GTPase as was yohimbine (see Fig. 3).

$[Ca^{2+}]_i$

Similarly to the results obtained in the high-affinity GTPase experiments, both phenylethylamine and non-phenylethylamine α_2-adrenoceptor agonists produced increases in $[Ca^{2+}]_i$ in HEL cells (Fig. 4). Adrenaline, α-methylnoradrenaline and UK 14304 were the most effective, followed by BHT 920 and *p*-amino clonidine. Clonidine, oxymetazoline and cirazoline did not produce any significant increase in $[Ca^{2+}]_i$. The increases in $[Ca^{2+}]_i$ produced by adrenaline and UK 14304 were concentration dependent, with EC_{50} values of 294 ± 32 nM and 65 ± 31 nM, respectively (means ± SD of two to three independent experiments performed in duplicate). The UK 14304-induced increases in $[Ca^{2+}]_i$ were potently inhibited by adrenergic drugs with the following order of potency: yohimbine ≈ oxymetazoline > > ARC-239 (see Fig. 4). Prazosin at 1 μM did not inhibit the UK 14304-

Table 1. Comparison of α_2-adrenoceptors on HEL cells with defined subtypes of α_2-adrenoceptors

Cell type	HEL		Platelets (α_{2A})	NG108–15 cells (α_{2B})	OK cells (α_{2C})	RINm5F cells (α_{2D})
Antagonist	pK_i	pK_B	pK_i	pK_i	pK_i	pK_i
Yohimbine	8.82 ± 0.38	8.49 ± 0.02	9.0	9.17	9.72	6.9
Oxymetazoline	8.30 ± 0.19	8.06 ± 0.04	9.09	7.4	7.5	7.39
ARC-239	6.00 ± 0.12	6.07 ± 0.06	7.06	8.77	7.89	5.72
Prazosin	5.88 ± 0.10	nd	6.5	8.43	7.8	5.25
Clonidine	nd*	7.01 ± 0.64				
Cirazoline	nd	6.60 ± 0.13				
Oxy/Yoh†	3.3	2.69	1	57	53	0.025
Praz/Yoh	871	nd	~1000	5	40	18

Values from this work are means ± SD of two to three experiments with different batches of HEL membranes performed in duplicate (pK_i), or means ± SD of two to three experiments with different batches of HEL cells performed in duplicate (pK_B). Data for defined receptors come from Refs. [26] (α_{2A}, α_{2B}), [27] (α_{2C}) and [30] (α_{2D}).

* nd, not determined; † ratios of K_i values for oxymetazoline (Oxy), yohimbine (Yoh), prazosin (Praz) and yohimbine (Yoh), respectively.

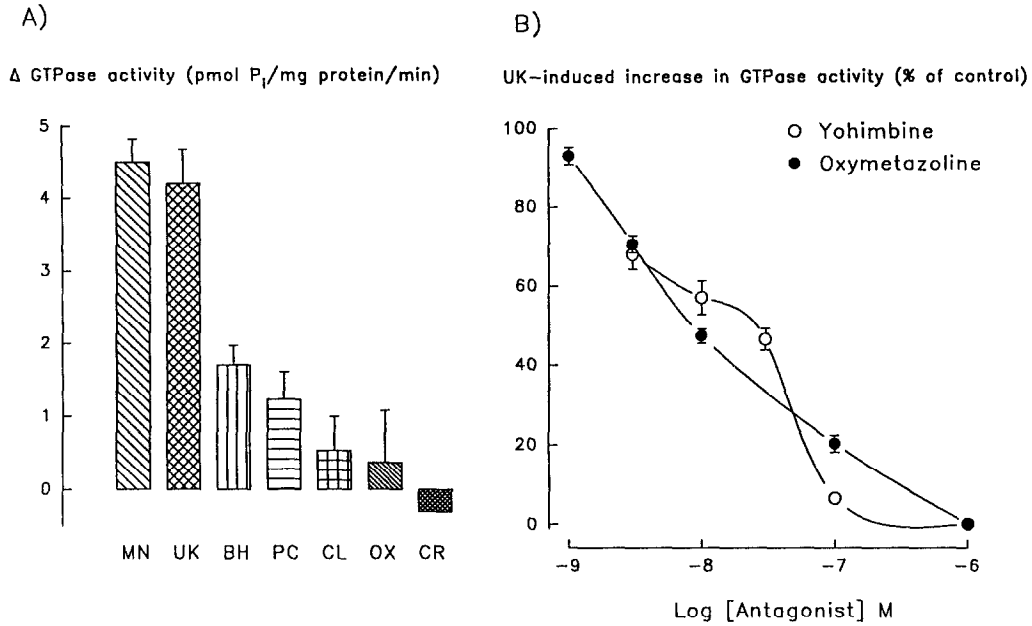

Fig. 3. Effects of adrenergic drugs on high-affinity GTPase activity. A: Effects of adrenergic drugs on high-affinity GTPase were determined as described in Materials and Methods. All drugs were used at the maximally effective concentration (100 μM). α-Methylnoradrenaline (MN) and UK 14304 (UK) were more effective than BHT 920 (BH) and *p*-amino clonidine (PC), while oxymetazoline (OX), clonidine (CL) and cirazoline (CR) produced no significant increase in high-affinity GTPase activity. Basal activity was 19.1 ± 1.0 pmol P_i/mg protein/min. B: Effect of yohimbine and oxymetazoline at increasing concentrations on the increase in GTPase activity induced by UK 14304 (1 μM), expressed as percentage of control. The data represent means ± SD of a typical experiment performed in quadruplicate. When error bars are smaller than the symbol size they are not shown. Similar results were seen in at least two further experiments.

induced increases in $[Ca^{2+}]_i$. Unfortunately, higher concentrations of prazosin produced significant autofluorescence and could not be used. Calculated pK_B values for yohimbine, oxymetazoline and ARC-239 are shown in Table 1. Clonidine and cirazoline also acted as antagonists (pK_B values shown in Table 1). Oxymetazoline inhibited the adrenaline-induced increase in $[Ca^{2+}]_i$ with a pK_B of 8.62 ± 0.01 (mean ± SD of two independent experiments performed in duplicate), similar to that found with UK 14304 (see Table 1). Clonidine and oxymetazoline (100 μM) had no effect on the increases in $[Ca^{2+}]_i$ induced by ATP (1 μM: 88 ± 14 and 72 ± 21% of control, respectively, N = 2), or thrombin (0.1 U/mL, 90 ± 10 and 94 ± 9% of control, respectively, N = 2). The UK 14304-induced increases in $[Ca^{2+}]_i$ were also completely blocked by RX821002 and idazoxan (1 μM, data not shown).

The α_2-adrenoceptor agonist-induced increases in $[Ca^{2+}]_i$ were abolished by pretreatment with pertussis toxin (see Table 2), and consisted of a small intracellular release component and a large extra-

Table 2. Effect of pertussis toxin pretreatment on adrenergic agonist-induced activation of high-affinity GTPase and stimulation of increases in $[Ca^{2+}]_i$

	ΔGTPase activity (pmol P_i/mg protein/min)		Δ$[Ca^{2+}]_i$ (nM)	
	Vehicle	Pertussis toxin	Vehicle	Pertussis toxin
Adrenaline (100 μM)	nd*	nd	534 ± 24	0 ± 0
α-Methylnoradrenaline (100 μM)	7.5 ± 0.1	−0.4 ± 0.1	967 ± 178	10 ± 1
UK 14304 (10 μM)	4.5 ± 0.1	−1.0 ± 0.1	340 ± 63	−19 ± 1

Values are means ± SD of a typical experiment. GTPase assays were performed in quadruplicate. $[Ca^{2+}]_i$ measurements were performed in duplicate. Basal GTPase activity was 31.9 ± 0.2 and 14.6 ± 0.1 pmol P_i/mg protein/min for membranes from vehicle- and pertussis toxin-pretreated cells, respectively. Basal $[Ca^{2+}]_i$ was 189 ± 16 nM and 204 ± 5 nM for vehicle- and pertussis toxin-pretreated cells, respectively.

* nd, not determined.

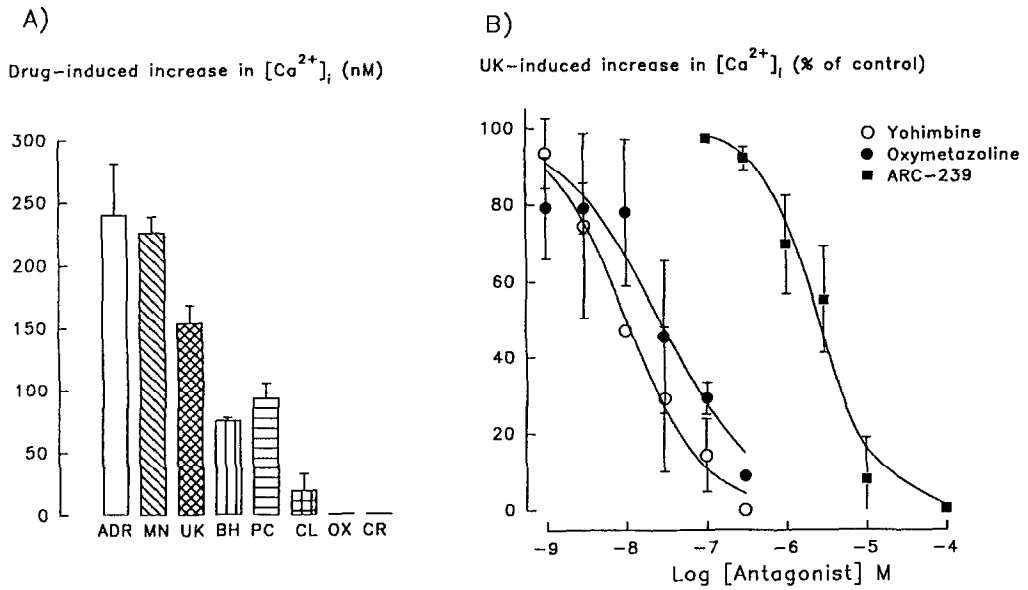

Fig. 4. Effects of adrenergic drugs on $[Ca^{2+}]_i$. A: Adrenergic drug-induced increases in $[Ca^{2+}]_i$. All drugs were used at a concentration of 100 μM except UK 14304 (10 μM). These concentrations were maximally effective. Adrenaline (ADR), α-methylnoradrenaline (MN) and UK 14304 (UK) were more effective than BHT 920 (BH) and p-amino clonidine (PC), while oxymetazoline (OX), clonidine (CL) and cirazoline (CR) produced no significant increase in $[Ca^{2+}]_i$. Basal $[Ca^{2+}]_i$ was 188 ± 30 nM. B: Effect of yohimbine, oxymetazoline and ARC-239 at increasing concentrations on the increase in $[Ca^{2+}]_i$ induced by UK 14304 (0.1 μM). Data represent means ± SD of two to three separate experiments performed in duplicate. When error bars are smaller than the symbol size they are not shown.

cellular entry component (Fig. 5). The extracellular entry component was effectively blocked by SKF 96365, a blocker of voltage-dependent calcium channels and NSC channels [25], but not by nitrendipine, a blocker of voltage-dependent calcium channels (Fig. 5). Furthermore, adrenaline produced quenching of fura-2 fluorescence in the presence of extracellular Mn^{2+} (50 μM, Fig. 6).

DISCUSSION

α_2-Adrenoceptors have been subdivided on the basis of antagonist potencies and DNA sequence data into four subtypes: α_{2A}, α_{2B}, α_{2C} and α_{2D} [1, 2]. The α_2-adrenoceptor of platelets is the prototypical α_{2A}-subtype [26, 27]. HEL cells have been used as a model for platelets and have been reported to have α_2-adrenoceptors [7, 8]. However, the α_2-adrenoceptor subtype of these cells is still unclear. Binding studies on membranes of our clone of HEL cells show that they have sites which bind the selective α_2-adrenoceptor antagonist [^{3}H]RX821002 saturably and specifically (see Fig. 1). [^{3}H]RX821002 is a recently developed α_2-adrenoceptor antagonist ligand with reported higher affinity and better specificity for α_2-adrenoceptors than [^{3}H]idazoxan or [^{3}H]yohimbine [28]. [^{3}H]RX821002 binds all described subtypes of α_2-adrenoceptors with low non-specific binding [28, 29] and has negligible affinity for imidazoline binding sites [30]. Displacement of [^{3}H]RX821002 by drugs had the order of potency yohimbine ≈ oxymetazoline > > ARC 239 = prazosin (see Fig. 2). The drugs used in the present study are reported to give the highest discrimination between the α_{2A}-adrenoceptor and the other described α_2-adrenoceptor subtypes [26, 27, 30]. The pK_i values of the drugs in HEL membranes correlated best with the published pK_i values for the α_{2A}-adrenoceptor of platelets. Furthermore, the ratios of the K_i values of oxymetazoline and prazosin to that of yohimbine, a more robust indicator of α_2-adrenoceptor subtype identity [30], also suggest that HEL cells have an adrenoceptor of the α_{2A} subtype ([30]; see also Table 1). Similarly, the pK_i values of oxymetazoline, ARC-239 and prazosin in HEL membranes were closer to their pK_i values vs [^{3}H]RX821002 at the α_2-adrenoceptor on CHO-C10 cells than for other subtypes (cf. values in [29]).

Binding sites have been described recently which have high affinity for drugs of the imidazoline and oxazoline classes previously thought to be selective for α_2-adrenoceptors, but do not bind phenylethylamines (for reviews see Refs. 11–13). These imidazoline binding sites are present on platelets [10, 31], and HEL cells have also been reported to have an excess of [^{3}H]idazoxan binding sites to [^{3}H]yohimbine binding sites [10], suggesting the presence of imidazoline receptors. The results of the present study strengthen this evidence. In the presence of 10 μM adrenaline, to block α_2-adrenoceptors, [^{3}H]idazoxan produced specific, saturable binding. The B_{max} and K_D of [^{3}H]idazoxan binding reported here are similar to those reported for [^{3}H]idazoxan binding sites in rat hippocampus [22] and rat adipocytes [32]. Unfortunately, similarly

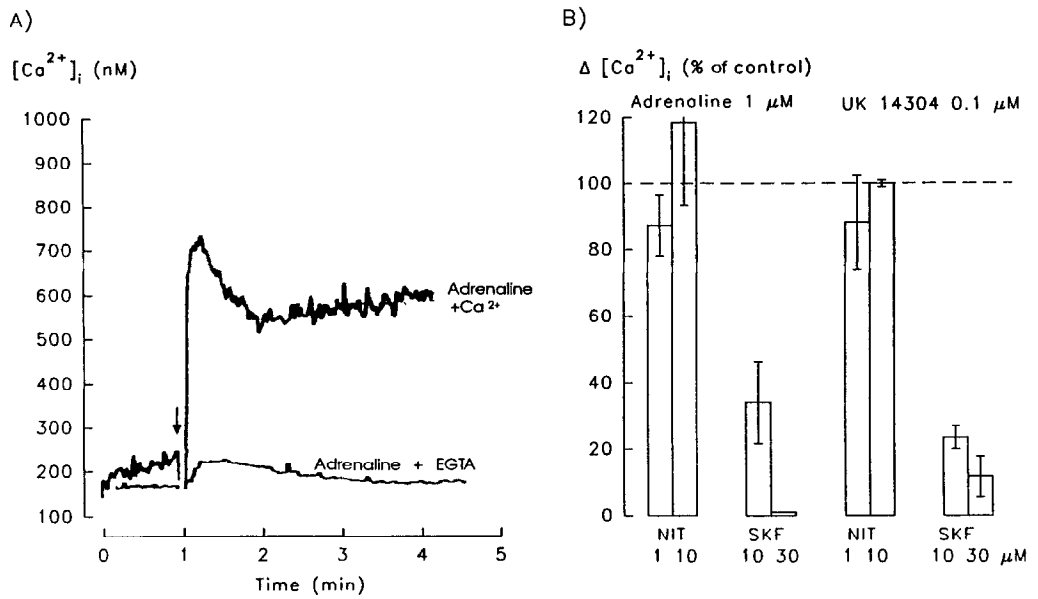

Fig. 5. Effect of adrenergic drugs on $[Ca^{2+}]_i$ in HEL cells. A: The effect of adrenaline (1 μM) on $[Ca^{2+}]_i$ in HEL cells in the presence of 1 mM extracellular Ca^{2+} or 1 mM extracellular EGTA. The arrow indicates the addition of adrenaline. Original traces of an experiment typical of at least three independent experiments performed in duplicate are shown. B: Effect of nitrendipine (NIT) or SKF 96365 (SKF) on the increases in $[Ca^{2+}]_i$ produced by adrenaline or UK 14304. Data represent the means ± SD of three separate experiments performed in duplicate.

to Brown *et al.* [22], the low binding values precluded further characterization of these sites.

Several α_2-adrenergic agonists stimulated high-affinity GTPase, indicating stimulation of G-proteins. α-Methylnoradrenaline, a phenylethylamine, and UK 14304, an imidazoline, were the most effective agonists (see Fig. 3). BHT 920, an azepine and *p*-amino clonidine, an imidazoline, were about 40% as effective as α-methylnoradrenaline and UK 14304 at E_{max}. Surprisingly, the partial α_2-adrenergic agonist clonidine and the α_{2A}-adrenoceptor-selective partial agonist oxymetazoline were ineffective at concentrations up to 100 μM. As clonidine and oxymetazoline also did not stimulate increases in $[Ca^{2+}]_i$ (see below), this suggests that this result is not due to a lack of sensitivity of the GTPase assay.

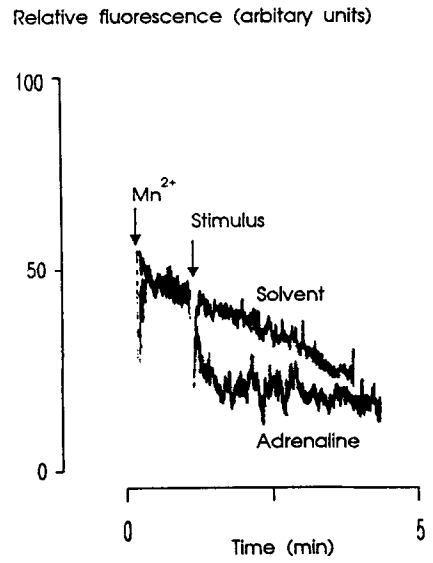

Fig. 6. Effect of adrenaline on Mn^{2+}-induced quenching of fura-2 fluorescence. Original tracings of a typical experiment performed in duplicate are shown. The top tracing represents Mn^{2+}-induced quenching of fura-2 fluorescence in the presence of solvent (control); the bottom tracing represents Mn^{2+}-induced quenching in the presence of adrenaline (10 μM). The concentration of Mn^{2+} was 50 μM. Arrows indicate the addition of the substances.

Oxymetazoline and clonidine also have high-affinity for one of the two broad classes of imidazoline binding sites [11–13], and we speculated that the lack of stimulation of high-affinity GTPase activity could be due to an interaction between imidazoline binding sites and α_2-adrenoceptors, as suggested by Piletz *et al.* [31]. However, this possibility is unlikely. UK 14304 and *p*-amino clonidine also have modest and high-affinity, respectively, for imidazoline binding sites [30, 31, 33] as well as α_2-adrenoceptors, yet they produced significant stimulation of high-affinity GTPase. Furthermore, oxymetazoline was as potent as yohimbine as a blocker of UK 14304-induced stimulation of high-affinity GTPase (see Fig. 3). While the low increases of high-affinity GTPase activity prevented the accurate estimation of pK_B values, the relative potencies of yohimbine and oxymetazoline were similar to those in our binding studies, suggesting that oxymetazoline is simply acting as an α_{2A}-adrenoceptor antagonist. Cirazoline, an imidazoline α_2-adrenoceptor blocker which has a high affinity at several imidazoline binding sites [22, 30, 33] also had no effect on high-affinity GTPase.

The effects of both the phenylethylamine α-methylnoradrenaline and the imidazoline UK 14304 on high-affinity GTPase were both abolished by pretreatment with pertussis toxin (see Table 1). HEL cells are known to express pertussis toxin-sensitive G-proteins of the G_i family, in the order of abundance $G_{\alpha i2} >> G_{\alpha i3}$ [34]. This suggests that in HEL cells α_2-adrenoceptor agonists exert their effects through activating G_i-proteins, in agreement with a previous report [8].

Michel *et al.* [8] have previously reported that α_2-adrenergic agonists stimulated an increase in $[Ca^{2+}]_i$ due solely to release of Ca^{2+} from intracellular stores. We also observed α_2-adrenergic agonist-stimulated release of Ca^{2+} from intracellular stores, but in addition, we also observed substantial entry of extracellular Ca^{2+}. The pattern of efficacy for agonists was similar in our study and that of Michel *et al.* [8]. The receptors in their study were described as "α_{2A}-adrenoceptor-like" [8], suggesting that differences in receptor subtype are not responsible, and may lie in elements of the signal transduction cascade. The difference between our observations and that of Michel *et al.* [8] may be in part related to the source of HEL cells. We obtained our cells from the American Type Culture Collection, whereas Michel *et al.* [8] obtained cells directly from the originator of the cell line.

We characterized the Ca^{2+} entry mechanism in our clone of HEL cells. The increase in $[Ca^{2+}]_i$ was unaffected by high concentrations of nitrendipine, a blocker of L-type voltage-dependent calcium channels, but was blocked by SKF 96365, a blocker of both NSC channels and L-type voltage-dependent calcium channels [25]. This suggests that α_2-adrenoceptors are activating NSC channels in these cells. NSC channels, unlike voltage-dependent channels, are permeable to several cations such as Na^+, Ca^{2+} and Mn^{2+} under physiological conditions [20, 21, 35]. Significantly, activation of α_2-adrenoceptors on HEL cells stimulates Mn^{2+} entry, which also suggests activation of NSC channels [20]. Furthermore, we have also seen α_2-adrenoceptor-activated Ca^{2+} entry via NSC channels in the megakaryocyte-like DAMI cell line (unpublished data), indicating that this phenomenon is not confined to HEL cells. As NSC channels are a major source of agonist-induced Ca^{2+} influx in non-excitable cells these results have important implications for the functional role of α_2-adrenoceptors [35].

The stimulation of Ca^{2+} entry and mobilization by α_2-adrenoceptors in HEL cells is an apparent difference between HEL cells and platelets. However, although α_2-adrenoceptor agonists do not increase $[Ca^{2+}]_i$ in platelets when measured with quin-2, fura-2 or indo-1 [36–39], adrenaline-induced increases in $[Ca^{2+}]_i$ are consistently reported with the luminescent Ca^{2+} indicator protein aequorin [38–40]. The reasons for the differences between the results obtained with various fluorescent probes and aequorin appear to be due to compartmentalization, with aequorin responding preferentially to changes in Ca^{2+} near the plasma membrane [38–40]. Furthermore, adrenaline stimulates $[^{45}Ca^{2+}]$ uptake [41, 42] into human platelets via α_2-adrenoceptors [41], and the effects of adrenaline on platelet aggregation can be blocked by inorganic calcium channel blockers such as Gd^{3+} [43] and chelation of cytoplasmic Ca^{2+} [37]. Taken together the results suggest that under certain conditions α_2-adrenoceptor agonists may indeed stimulate Ca^{2+} influx into platelets, although on a more restricted scale than found in HEL cells.

α_2-Adrenoceptor agonists have been reported to activate NSC channels in several vascular smooth muscle preparations [44, 45]. This may explain their ability to increase $[Ca^{2+}]_i$ in vascular smooth muscle [3, 4]. The α_2-adrenoceptor subtype activating the NSC channel of vascular smooth muscle is unclear for most vascular beds. However, the dog saphenous vein possesses α_2-adrenoceptors which are most likely of the α_{2A}-subtype [46], and supports the utility of the HEL cell line as a model system.

The efficacy of α_2-adrenergic agonists in stimulating increases in $[Ca^{2+}]_i$ was very similar to that seen for stimulation of high-affinity GTPase activity (see Figs 3 and 4). Adrenaline, α-methylnoradrenaline and UK 14304 were the most effective agonists, while BHT 920 and *p*-amino clonidine had E_{max} values approximately 40–50% of that of α-methylnoradrenaline, as seen with high-affinity GTPase. Importantly, those drugs that did not stimulate high-affinity GTPase activity; clonidine, oxymetazoline and cirazoline also did not stimulate increases in $[Ca^{2+}]_i$, and were relatively potent antagonists. Also, as in the experiments on high-affinity GTPase activity, the effects of both phenylethylamine and imidazoline agonists were abolished by pretreatment with pertussis toxin (see Table 2). The lack of effect of prazosin (1 μM) on UK 14304-induced increases in $[Ca^{2+}]_i$ and the pK_B values of yohimbine, oxymetazoline, and ARC-239 are consistent with the pK_i values in our binding studies, and suggest that stimulation of increases in $[Ca^{2+}]_i$ is mediated through activation of α_{2A}-adrenoceptors, with no component due to imidazoline receptors. The pK_B values of clonidine and cirazoline are closer to values for α_{2A}-adrenoceptors than either of the two subdivisions of imidazoline binding sites (cf. K_i values in Refs. 8, 11, 22, 31, 33, 47). This suggests they are acting as α_2-adrenoceptor antagonists rather than inhibiting α_2-adrenoceptor-mediated increases in $[Ca^{2+}]_i$ through an action at imidazoline binding sites. A similar argument holds for the inhibitory effect of oxymetazoline. Furthermore, oxymetazoline was as potent at blocking the increases in $[Ca^{2+}]_i$ induced by the phenylethylamine adrenaline as those induced by the imidazoline UK 14304, again suggesting that oxymetazoline is acting as an α_2-adrenoceptor antagonist rather than interacting with imidazoline binding sites.

While it would appear unusual that the partial α_2-adrenergic agonists oxymetazoline and clonidine are instead α_2-adrenoceptor antagonists in HEL cells, this lack of agonism has been observed for oxymetazoline previously [8]. This is not simply a property of HEL cells, as similar results have been seen in the megakaryocyte DAMI cell line. Furthermore, in platelets, the archetypical α_{2A}-adrenoceptor-containing tissue, clonidine and oxymetazoline have no effect on aggregation or adenylate cyclase inhibition themselves, but are

blockers of adrenaline-induced aggregation and adrenaline-induced inhibition of adenylate cyclase activity [2, 36, 47]. Thus the α_{2A}-adrenoceptor of HEL cells also appears to have similar agonist efficacy to the α_{2A}-adrenoceptor of platelets.

While clonidine inhibits the adrenaline-induced platelet aggregation, it also potentiates the aggregation induced by other agents such as thrombin [47]. This action of clonidine has been suggested to be mediated by imidazoline binding sites [31]. As increases in $[Ca^{2+}]_i$ are thought to play an important role in the aggregatory response to thrombin [35, 37], we investigated whether clonidine or oxymetazoline could modulate the stimulation-induced increases in $[Ca^{2+}]_i$ produced by thrombin and ATP in HEL cells. Neither clonidine nor oxymetazoline potentiated the increases in $[Ca^{2+}]_i$ produced by thrombin or ATP in HEL cells. This suggests that the potentiating effect of imidazolines on thrombin-induced aggregation in platelets is not mediated via enhancement of increases in $[Ca^{2+}]_i$.

In conclusion, HEL cells appear to have a single population of α_{2A}-adrenoceptors, activation of which results in the stimulation of the high-affinity GTPase of G_i-proteins, which subsequently mediates the release of Ca^{2+} from intracellular stores and entry of Ca^{2+} through NSC channels. HEL cells are a widely used model of platelets [7–9]. The confirmation that they have the same α_2-adrenoceptor subtype as platelets, with a similar receptor density and a similar pattern of agonist efficacy, most especially that clonidine and oxymetazoline act as α_2-adrenoceptor antagonists in both HEL and platelets, confirms their utility as a model of platelets. While there is an apparent difference in the ability of α_2-adrenoceptors to stimulate Ca^{2+} entry in HEL cells and platelets [36–39], HEL cells may nonetheless give some useful insights into the role of Ca^{2+} in α_2-adrenoceptor-mediated effects in platelets. In addition, HEL cells may prove a useful model for studying α_2-adrenoceptor-mediated stimulation of Ca^{2+} entry, such as seen in vascular smooth muscle. HEL cells also possess an imidazoline binding site. However, we could find no evidence that it had either a direct or an indirect role on either stimulation of GTPase activity or modulation of $[Ca^{2+}]_i$.

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REFERENCES

1. Lomasney JW, Cotecchia S, Lefkowitz RJ and Caron MG, Molecular biology of α-adrenergic receptors: implications for receptor classification and for structure-function relationships. *Biochim Biophys Acta* **1095**: 127–139, 1991.
2. Ruffolo RRJ, Nichols AJ, Stadel JM and Hieble JP, Pharmacologic and therapeutic applications of α_2-adrenoceptor subtypes. *Annu Rev Pharmacol Toxicol* **33**: 243–279, 1993.
3. Jim KF and Matthews WD, Role of extracellular calcium in contractions produced by activation of postsynaptic α_2-adrenoceptors in the canine saphenous vein. *J Pharmacol Exp Ther* **234**: 161–165, 1985.
4. Aburto TK, Lajoie C and Morgan KG, Mechanisms of signal transduction during α_2-adrenergic receptor-mediated contraction of vascular smooth muscle. *Circ Res* **72**: 778–785, 1993.
5. Murray R, Furci L and FitzGerald GA, Induction of prostacyclin receptor expression in human erythroleukaemia cells. *FEBS Lett* **255**: 172–174, 1989.
6. Nakajima M, Yamamoto M, Ushikubi F, Okuma M, Fujiwara M and Narumiya S, Expression of thromboxane A_2 receptor in cultured human erythroleukaemia cells and its induction by 12-*O*-tetradecanoylphorbol-13-acetate. *Biochem Biophys Res Commun* **158**: 958–965, 1989.
7. McKernan RM, Howard MJ, Motulsky HJ and Insel PA, Compartmentation of α_2-adrenergic receptors in human erythroleukaemia (HEL) cells. *Mol Pharmacol* **32**: 258–265, 1987.
8. Michel MC, Brass LF, Williams A, Bokoch GM, LaMorte VJ and Motulsky HJ, α_2-adrenergic receptor stimulation mobilizes intracellular Ca^{2+} in human erythroleukaemia cells. *J Biol Chem* **264**: 4986–4991, 1989.
9. Schwaner I, Seifert R and Schultz G, Receptor-mediated increases in cytosolic Ca^{2+} in the human erythroleukaemia cell line involve pertussis toxin-sensitive and -insensitive pathways. *Biochem J* **281**: 301–307, 1992.
10. Michel MC, Brodde OE, Schnepel B, Behrendt J, Tschada R, Motulsky HJ and Insel PA, [^{3}H]idazoxan and some other α_2-adrenergic drugs also bind with high affinity to a nonadrenergic site. *Mol Pharmacol* **35**: 324–330, 1989.
11. Atlas D, Clonidine displacing substance (CDS) and its putative imidazoline receptor. *Biochem Pharmacol* **41**: 1541–1549, 1991.
12. Bousquet P, Feldman J, Tibirica E, Bricca G, Greney H, Dontenwill M, Stutzmann J and Belcourt A, Imidazoline receptors. A new concept in central regulation of the arterial blood pressure. *Am J Hypertens* **5**: 47S–50S, 1992.
13. Kilpatrick AT, Brown CC and MacKinnon AC, Non-α_2-adrenoceptor idazoxan binding sites; a new target for drug development. *Biochem Soc Trans* **20**: 113–118, 1992.
14. Seifert R and Schultz G, Fatty-acid-induced activation of NADPH oxidase in plasma membranes of human neutrophils depends on neutrophil cytosol and is potentiated by stable guanine nucleotides. *Eur J Biochem* **162**: 563–569, 1987.
15. Lowry OH, Rosenbrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
16. Cheng YC and Prusoff WH, Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22**: 3099–3108, 1973.
17. Johnson RA and Walseth TF, The enzymatic preparation of [α-^{32}P]-ATP, [α-^{32}P]GTP, [^{32}P]cAMP and [^{32}P]cGMP, and their use in the assay of adenylate and guanylate cyclases and cyclic nucleotide phosphodiesterases. *Adv Cyclic Nucleotide Res* **10**: 135–167, 1979.
18. Seifert R, Serke S, Huhn D, Bessler WG, Hauschildt S, Metzger J, Wismüller KH and Jung G, Incomplete functional differentiation of HL-60 leukemic cells by synthetic lipopeptides. Partial inhibition by pertussis toxin of enhanced superoxide formation. *Eur J Biochem* **203**: 143–151, 1992.
19. Grynkiewicz G, Poenie M and Tsien RY, A new

generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260**: 3440–3450, 1985.

20. Merritt JE, Jacob R and Hallam TJ, Use of manganese to discriminate between calcium influx and mobilization from internal stores in stimulated human neutrophils. *J Biol Chem* **264**: 1522–1527, 1989.
21. Musgrave IF, Seifert R and Schultz G, Maitotoxin activates cation channels distinct from the receptor-activated non-selective cation channels of HL-60 cells. *Biochem J* **301**: 437–441, 1994.
22. Brown CM, MacKinnon AC, McGrath JC, Spedding M and Kilpatrick AT, α_2-Adrenoceptor subtypes and imidazoline-like binding sites in the rat brain. *Br J Pharmacol* **99**: 803–809, 1990.
23. Milligan G, Techniques used in the identification and analysis of function of pertussis toxin-sensitive guanine nucleotide binding proteins. *Biochem J* **255**: 1–13, 1988.
24. Gierschik P and Jakobs KH, Receptor-stimulated GTPase activity of G-proteins. In: *G-proteins as Mediators of Cellular Signalling Processes* (Eds. Houslay MD and Milligan G), pp. 67–82. Wiley, Chichester, 1990.
25. Merritt JE, Armstrong WP, Benham CD, Hallam TJ, Jacob R, Jaxa Chamiec A, Leigh BK, McCarthy SA, Moores KE and Rink TJ, SK&F 96365, a novel inhibitor of receptor-mediated calcium entry. *Biochem J* **271**: 515–522, 1990.
26. Bylund DB, Ray Prenger C and Murphy TJ, *Alpha*-2A and *alpha*-2B adrenergic receptor subtypes: antagonist binding in tissues and cell lines containing only one subtype. *J Pharmacol Exp Ther* **245**: 600–607, 1988.
27. Bylund DB, Blaxall HS, Iversen LJ, Caron MG, Lefkowitz RJ and Lomasney JW, Pharmacological characteristics of α_2-adrenergic receptors: comparison of pharmacologically defined subtypes with subtypes identified by molecular cloning. *Mol Pharmacol* **42**: 1–5, 1992.
28. Galitzky J, Senard JM, Lafontan M, Stillings M, Montastruc JL and Berlan M, Identification of human platelet α_2-adrenoceptors with a new antagonist [^{3}H]-RX821002, a 2-methoxy derivative of idazoxan. *Br J Pharmacol* **100**: 862–866, 1990.
29. O'Rourke MF, Blaxall HS, Iversen, LJ and Bylund DB, Characterization of [^{3}H]RX821002 binding to *alpha*-2 adrenergic receptor subtypes. *J Pharmacol Exp Ther* **268**: 1362–1367.
30. Remaury A and Paris H, The insulin-secreting cell line, RINm5F, expresses an *alpha*-2D adrenoceptor and nonadrenergic idazoxan-binding sites. *J Pharmacol Exp Ther* **260**: 417–426, 1992.
31. Piletz JE, Andorn AC, Unnerstall JR and Halaris A, Binding of [^{3}H]-*p*-aminoclonidine to α_2-adrenoceptor states plus a non-adrenergic site on human platelet plasma membranes. *Biochem Pharmacol* **42**: 569–584, 1991.
32. Carpéné C, Galitzky J, Larrouy D, Langin D and Lafontan M, Non-adrenergic sites for imidazolines are not directly involved in the α_2-adrenergic antilipolytic effect of UK 14304 in rat adipocytes. *Biochem Pharmacol* **40**: 437–445, 1990.
33. Molderings GJ, Moura D, Fink K, Bönisch H and Göthert M, Binding of [^{3}H]clonidine to I_1-imidazoline sites in bovine adrenal medullary membranes. *Naunyn-Schmiedebergs Arch Pharmacol* **348**: 70–76, 1993.
34. Williams AG, Woolkalis MJ, Poncz M, Manning DR, Gewirtz AM and Brass LF, Identification of the pertussis toxin-sensitive G proteins in platelets, megakaryocytes, and human erythroleukaemia cells. *Blood* **76**: 721–730, 1990.
35. Sage S. Mahaut-Smith MP and Rink TJ, Calcium entry in nonexcitable cells: lessons from human platelets. *News Physiol Sci* **7**: 109–113, 1992.
36. Ruffolo RR, Nichols AJ and Hieble JP, Functions mediated by *alpha*-2 adrenergic receptors. In: *The Alpha-2 Adrenergic Receptors* (Ed. Limbird LE), pp. 187–249. Humana Press, Clifton, NJ, 1989.
37. Siess W, Molecular mechanisms of platelet activation. *Physiol Rev* **69**: 58–178, 1989.
38. Ware JA, Smith M and Salzman EW, Synergism of platelet-aggregating agents. *J Clin Invest* **80**: 267–271, 1987.
39. Johnson PC, Ware JA and Salzman EW, Measurement of platelet cytoplasmic ionized calcium concentration with aequorin and fluorescent indicators. *Methods Enzymol* **169**: 386–415, 1989.
40. Lages B and Weiss HJ, Time dependence of aequorin-indicated calcium levels in stimulated and unstimulated platelets: evidence for multiple aequorin environments in platelets. *Thromb Haemost* **62**: 1094–1099.
41. Gill J, DeSouza V, Wakeling A, Dandona P and Jeremy JY, Differential changes in alpha- and beta-adrenoceptor linked [$^{45}Ca^{2+}$] uptake in platelets from patients with anorexia nervosa. *J Clin Endocrinol Metab* **74**: 441–446, 1992.
42. Jeremy JY, Gill J and Mikhailidis D, Effect of milrinone on thromboxane A2 synthesis, cAMP phosphodiesterase and $^{45}Ca^{2+}$ uptake by human platelets. *Eur J Pharmacol* **245**: 67–73, 1993.
43. Blache D, Ciavatti M and Ojeda C, The effect of calcium channel blockers on blood platelet function, especially calcium uptake. *Biochim Biophys Acta* **932**: 401–412, 1987.
44. Lee JY, Warner RB, Brune ME and DeBernardis JF, Calcium-induced vasocontractions after α_2-adrenergic receptor activation in the dog saphenous vein: comparison to calcium-induced contractions after potassium-depolarization. *Gen Pharmacol* **21**: 647–654, 1990.
45. Wang Q and Large WA, Noradrenaline-evoked cation conductance recorded with the nystatin whole-cell method in rabbit portal vein cells. *J Physiol Lond* **435**: 21–39, 1991.
46. Hicks PE, Barras M, Herman G, Mauduit P, Armstrong JM and Rossignol B, *Alpha*-adrenoceptor subtypes in dog saphenous vein that mediate contraction and inositol phosphate production. *Br J Pharmacol* **102**: 151–161, 1991.
47. Clare KA, Scrutton MC and Thompson NT, Effects of α_2-adrenoceptor agonists and of related compounds on aggregation of, and on adenylate cyclase activity in, human platelets. *Br J Pharmacol* **82**: 467–476, 1984.