

Regulation of $[Ca^{++}]_i$ in Human Neuroblastoma (SH-SY5Y) Cells Expressing Recombinant Rat Angiotensin $_{1A}$ Receptors by Angiotensin II and Carbachol¹

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ABSTRACT

The ability of angiotensin II (All) to regulate $[Ca^{++}]_i$ in human neuroblastoma (SH-SY5Y) cells stably expressing recombinant rat AT $_{1A}$ receptors was investigated using microfluorimetric methods, and compared to responses obtained by stimulation of native muscarinic receptors. Applications of All or carbachol produced biphasic rises of $[Ca^{++}]_i$, but in Ca $^{++}$ -free solutions (containing 1 mM ethylene glycol-bis (β -aminoethyl ether)N,N,N',N'-tetraacetic acid), both agonists produced only transient monophasic rises of $[Ca^{++}]_i$, and second applications were without effect. Application of Ca $^{++}_o$ (2.5 mM) to cells after exposure to either agonist produced a Ni $^{2+}$ -sensitive rise of $[Ca^{++}]_i$ in the absence of agonist ("capacitative Ca $^{++}$ influx"). After removal of Ca $^{++}_o$, both All and carbachol elicited a sec-

ond rise of $[Ca^{++}]_i$. Thapsigargin (1 μ M) prevented these second rises of $[Ca^{++}]_i$. During capacitative Ca $^{++}$ influx, application of All failed to produce a further rise of $[Ca^{++}]_i$. In contrast, carbachol produced a further rise of $[Ca^{++}]_i$, attributable to activation of both nicotinic and muscarinic receptors, because it was reduced (but not abolished) by mecamylamine (1 μ M) and was observed when muscarine was used as the agonist. Thus, activation of recombinant AT $_{1A}$ and muscarinic receptors in SH-SY5Y cells leads to mobilization of Ca $^{++}$ from a common intracellular pool, and stimulates capacitative Ca $^{++}$ influx. Muscarinic (but not All) receptor occupancy is capable of stimulating an additional Ca $^{++}$ influx pathway.

All is an octapeptide hormone of the renin-angiotensin system that is involved in cardiovascular homeostasis and may contribute to pathological situations such as hypertension (Reid, 1992). All evokes pressor responses by acting centrally to increase sympathetic outflow (Matsukawa and Reid, 1990), stimulating catecholamine release from the adrenal medulla (Peach et al., 1966) and facilitating NA release from sympathetic nerve terminals. This latter effect on NA release has been demonstrated to occur by two separate mechanisms: first, All can act at postsynaptic cell bodies within sympathetic ganglia to enhance NA release in a manner comparable to that of muscarinic agonists (Brown et al., 1980). This effect is believed to be mediated by AT $_1$ receptors (Wong et al., 1990). Second, All can act presynaptically at postganglionic sympathetic nerve terminals to enhance NA release (Zimmermann et al., 1987).

The cellular mechanisms underlying enhancement of NA release by All remain to be fully elucidated, and AT $_1$ recep-

tors are known to couple to different signal transduction pathways in various cell types (Timmermans et al., 1993). However, it is likely that All regulates NA release by regulating $[Ca^{++}]_i$ in sympathetic neurons. To investigate this, we have examined the regulation of intracellular calcium ($[Ca^{++}]_i$) by All in SH-SY5Y cells stably transfected with the rat AT $_{1A}$ receptor. We have compared All-mediated responses to the well-described responses evoked by stimulation of native muscarinic (M $_3$) receptors (Murphy et al., 1991a,b; Purkiss et al., 1995; Grudt et al., 1996). The neuroblastoma SH-SY5Y is derived from human sympathetic tissue and retains many of the properties of sympathetic neurones (Vaughan et al., 1995b). These cells possess a wide variety of receptors (Vaughan et al., 1995b), are electrically excitable (Vaughan et al., 1995a) and, perhaps most importantly, retain the ability to synthesise and release NA in a Ca $^{++}$ -dependent manner in response to various stimuli (Murphy et al., 1991a; Vaughan et al., 1995b). Recently, we have shown that All evokes NA release in SH-SY5Y cells stably transfected with rat AT $_{1A}$ receptors, and that this effect is only partially dependent on the presence of extracellular Ca $^{++}$ (McDonald et al., 1995a). Such a finding is qual-

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ABBREVIATIONS: All, angiotensin II; EGTA, ethylene glycol-bis(β -aminoethyl ether)N,N,N',N'-tetraacetic acid; PBS, phosphate-buffered saline; HEPES, (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]); AM, acetoxy methylester; NA, noradrenaline; DMPP, dimethylphenylpiperazine; $[Ca^{++}]_i$, intracellular calcium concentration; IP, inositol phosphate.

itatively similar to the actions of muscarinic agonists acting at M_3 receptors in these cells (Murphy *et al.*, 1991a,b; Purkiss *et al.*, 1995), although AII, like as bradykinin (McDonald *et al.*, 1994a), is a less effective secretagogue than muscarinic agonists (Murphy *et al.*, 1991a). We demonstrate that both AII and carbachol can mobilize Ca^{++} from the same intracellular stores. Both agonists are also capable of stimulating capacitative Ca^{++} entry. However, unlike activation of muscarinic receptors, AII receptor activation is not able to evoke an additional Ca^{++} influx pathway that is dependent on receptor occupancy. A preliminary account of some of these findings has been published (McDonald *et al.*, 1995b).

Methods

Transfection and culture of SH-SY5Y cells. The human neuroblastoma SH-SY5Y (kindly provided by Dr June L. Biedler of the Sloan-Kettering Institute for Cancer Research, Rye, NJ) was stably transfected with a mammalian expression plasmid (pCEP4) containing the full length rat AT_{1A} cDNA. The transfection and selection procedures for SH-SY5Y cells expressing AT_{1A} receptors were performed exactly as previously described for human embryonic kidney (HEK 293) cells (Balmforth *et al.*, 1995). A stable cell line expressing the AT_{1A} receptor was cultured in a 1:1 mixture of Ham's F12 and Eagle's minimal essential medium containing nonessential amino acids, 300 μ g/ml hygromycin (to select and maintain the transfected cell lines) and 10% fetal calf serum. The cells were grown in a 98% air, 2% CO_2 humidified incubator at 37°C. When confluent, cell layers were harvested from flasks by gentle agitation following incubation at 37°C for 10 min in Ca^{++} - and Mg^{++} -free PBS in the absence of trypsin, to select against possible contamination of epithelioid SH-EP cells (Ciccarone *et al.*, 1989; Ogino and Costa, 1992). The cell suspension was triturated using a 0.8-mm gauge needle and the cell number estimated using a Coulter Counter (Coulter Electronics Ltd., Luton, U.K.). Cells were plated onto glass coverslips in 35-mm Petri dishes at a seeding density of 0.3×10^5 cells/ml. These dishes were maintained in the incubator and cells adhered to coverslips and were grown for 3 to 6 days. No chemicals were added to induce cell differentiation. For experiments using nominally Ca^{++} -free solutions, coverslips were coated with polylysine to prevent detachment of cells during perfusion.

Determination of AII binding in transfected SH-SY5Y cells. Cells were seeded at 0.3×10^5 cells/ml and grown to 50% confluence in 12-well plates. Culture medium was replaced with 1ml per plate of minimum essential medium containing 20 mM HEPES, 0.25% w/v bacitracin, 0.25% w/v bovine serum albumin and [3H]AII (0.5–50 nM) for 25 min at 37°C (pH 7.4). Unbound ligand was then removed by twice rinsing cells with 4 ml of chilled phosphate-buffered saline. Receptors were solubilized overnight at room temperature in 1ml 0.1 M NaOH with 2% sodium dodecylsulfate. After neutralization with an equal volume of 0.1 M HCl, samples were measured for radioactivity by liquid scintillation counting. Samples of each well were also used for determination of total protein content by the BCA method. Nonspecific binding was determined in separate wells by supplementing [3H]AII samples with 10 μ M unlabeled AII. Total and nonspecific binding was determined at each [3H]AII concentration in duplicate on four separate occasions.

Measurement of $[Ca^{2+}]_i$ (intracellular calcium concentration) in SH-SY5Y cells. SH-SY5Y cells attached to glass coverslips were rinsed twice with 1ml HEPES-buffered saline (135 mM NaCl, 5 mM KCl, 0.6 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM HEPES and 6 mM glucose, pH 7.4) and incubated with 1 ml HBS containing 4 μ M Fura2/AM for 1 hr at room temperature. Fragments of the glass coverslip were placed in a 80- μ l perfusion chamber on the stage of an inverted Nikon microscope. The cells were continuously perfused at a rate of 10ml/min and each recording was taken from groups of approximately five cells. Changes in $[Ca^{++}]_i$ were measured from the

fluorescence emitted at 510 nm due to alternate excitation at 340 and 380 nm using Joyce Loebel PhoCal (Applied Imaging, Sunderland, U.K.) apparatus. The ratio, R, of the fluorescence at these wavelengths was converted to $[Ca^{++}]_i$ using the equation $[Ca^{++}]_i = K_d \beta [(R - R_{min}) / (R_{max} - R)]$ (see Grynkiewicz *et al.*, 1985). β is the ratio of fluorescence intensity at 380nm under maximal and minimal saturation of Fura-2 with Ca^{++} . R_{max} (1.72) was the fluorescence ratio observed when the dye was saturated with Ca^{++} ; this was obtained when the cell layers were exposed to 2 μ M ionomycin and R_{min} (0.55) represents the fluorescence ratio for free dye. Changes in $[Ca^{++}]_i$ were recorded continuously following perfusion of the cells with AII or carbachol. Results are expressed as mean \pm S.E.M. and statistical comparisons are made using the unpaired Student's *t* test.

Results

To ascertain whether transfection of SH-SY5Y cells was successful, radiolabeled AII binding studies were performed as described in "Methods." Scatchard analysis revealed a B_{max} value of 6.3 ± 0.3 pmol/mg protein, and a K_d of 3.0 ± 0.6 nM ($n = 4$). By contrast, in untransfected cells B_{max} was ≤ 10 fmol/mg protein in each of three separate determinations. Thus we were able to successfully establish a stable SH-SY5Y cell line expressing AT_{1A} receptors.

We have previously reported that bath application of AII (0.3–300 nM) was without effect on $[Ca^{++}]_i$ in untransfected SH-SY5Y cells, but caused concentration-dependent increases of $[Ca^{++}]_i$ in transfected cells which could be fully blocked by losartan (McDonald *et al.*, 1995a). Activation of muscarinic receptors by carbachol is also known to raise $[Ca^{++}]_i$ in SH-SY5Y (Lambert and Nahorski, 1990, 1992; Murphy *et al.*, 1991b; Forsythe *et al.*, 1992; Grudt *et al.*, 1996; Connor and Henderson, 1996). Figures 1, A and B illustrate in transfected SH-SY5Y cells that activation of both receptor types can evoke increases of $[Ca^{++}]_i$ in the same cells regardless of whether the cells had been preexposed to the other agonist; responses to 10 nM AII could be evoked after exposure to 100 μ M carbachol (fig. 1A, representative of three experiments) and vice versa (fig. 1B, representative of three experiments). Prolonged exposure to either carbachol (100 μ M) (fig. 1C) or AII (10 nM) (fig. 1D) produced an initial rapid rise of $[Ca^{++}]_i$ which declined to an elevated plateau level until agonist removal. These biphasic responses to either agonist are similar to previous reports describing the actions of muscarinic agonists (Lambert and Nahorski, 1990, 1992; Murphy *et al.*, 1991b; Grudt *et al.*, 1996; Connor and Henderson, 1996), but contrast markedly with experiments conducted in the absence of Ca^{++}_o , as illustrated in figure 2. In these Ca^{++} -free solutions ($CaCl_2$ replaced by 1 mM EGTA), 10 nM AII evoked rapid rises in $[Ca^{++}]_i$ and the peak levels (516 ± 45 nM, $n = 5$) were not significantly different to those observed in Ca^{++} -containing solutions. However, the pattern of response was different in that the rises in $[Ca^{++}]_i$ were transient and always fell to basal levels within 3 min (*i.e.*, no plateau phase was observed, *e.g.*, fig. 2A). Second applications of AII in the absence of extracellular Ca^{++} consistently failed to evoke a rise in $[Ca^{++}]_i$ ($n = 5$, *e.g.*, fig. 2A). Similarly in Ca^{++} -free solutions, 100 μ M carbachol evoked a transient rise in $[Ca^{++}]_i$ with a peak level of 688 ± 42 nM ($n = 5$) and, as for AII, second applications of carbachol failed to evoke any rise of $[Ca^{++}]_i$ (*e.g.*, fig. 2B). After application of carbachol in Ca^{++} -free solutions, subsequent applications of 10 nM AII were without effect on $[Ca^{++}]_i$ (fig. 2C, representa-

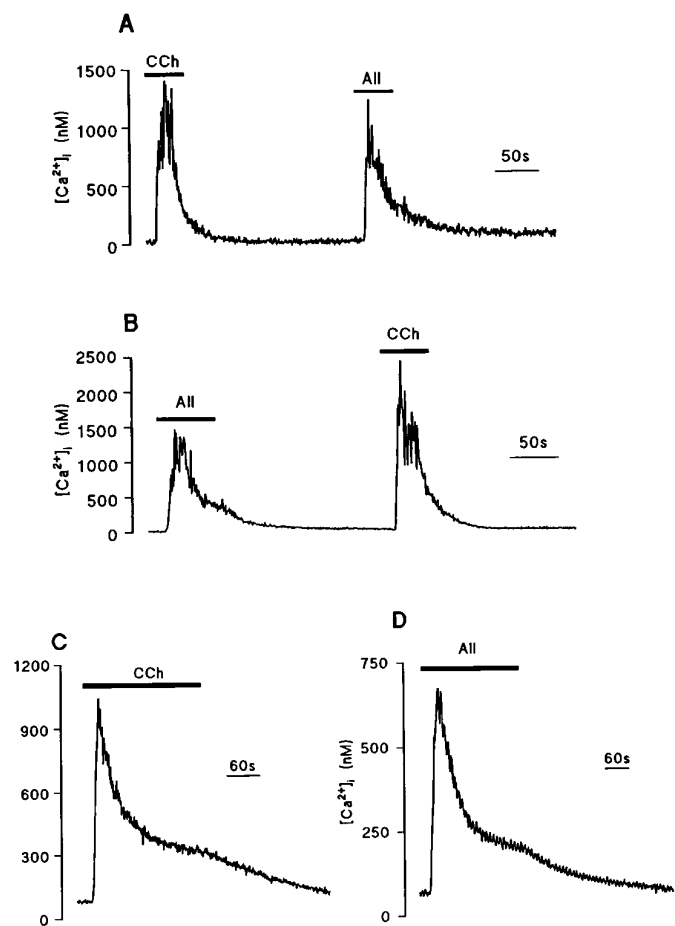


Fig. 1. (A) Measurement of $[Ca^{2+}]_i$ in a group of approximately five SH-SY5Y cells. Periods of exposure to 100 μ M carbachol (CCh) and 10 nM angiotensin II (AII) indicated by the solid bars. (B) As in (A), but 10 nM AII was applied before 100 μ M carbachol. (C) Prolonged exposure to 100 μ M carbachol produced a biphasic rise of $[Ca^{2+}]_i$, and a similar response was seen when AII was applied to cells (D).

tive of four recordings) and prior exposure of cells to 10 nM AII prevented responses to carbachol ($n = 5$), as exemplified in figure 2D.

It is apparent from figures 1 and 2 that the plateau phase of raised $[Ca^{2+}]_i$ seen in the presence of AII arises from Ca^{2+} influx across the plasma membrane. To determine whether this influx seen in Ca^{2+} -containing solutions was due to activation of voltage-gated L-type Ca^{2+} channels, the long exposures to 10 nM AII in Ca^{2+} -containing solutions were repeated in the presence of 5 μ M nifedipine or 2 μ M (–)Bay K 8644. No significant effect was found on the peak or plateau responses in the presence of either drug (McDonald, R. L., Vaughan, P. F. T. and Peers, C.: unpublished observations), although Bay K 8644 did cause a significant ($P < .002$) rise in basal levels to 208 ± 29 nM ($n = 5$).

After application and then removal of either carbachol or AII to SH-SY5Y cells in Ca^{2+} -free solutions, exposure to a Ca^{2+} -containing solution (2.5 mM) caused a rise of $[Ca^{2+}]_i$ which declined gradually in the continued presence of Ca^{2+} (fig. 3, A and B). Such a rise was not observed on replacement of Ca^{2+} if agonist had not previously been applied. This suggested that after washout of agonist, a Ca^{2+} -entry pathway was activated. After 100 μ M carbachol, the application of Ca^{2+} caused a rise of $[Ca^{2+}]_i$ to 246 ± 30 nM ($n = 3$, e.g.,

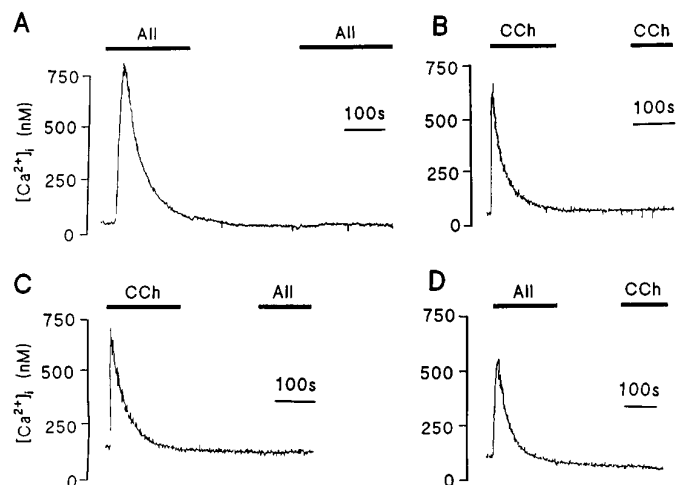


Fig. 2. $[Ca^{2+}]_i$ measurements in groups of approximately five SH-SY5Y cells. In all traces, extracellular Ca^{2+} (2.5 mM) was replaced by 1 mM EGTA. Agonist application periods indicated by solid bars. Cells were exposed twice to AII (A), or twice to carbachol (CCh; B). In (C), cells were exposed to CCh (100 μ M) and then AII (10 nM, and in (D) AII was applied before CCh. In all cases, CCh concentration was 100 μ M and AII concentration was 10 nM.

fig. 3A) and to 190 ± 5 nM after AII ($n = 3$, e.g., fig. 3B), values that were not significantly different. The rise of $[Ca^{2+}]_i$ observed on readdition of Ca^{2+} after store depletion by AII was also observed with losartan (2 μ M) present in the perfusate ($n = 5$, Roberts, D. J., Vaughan, P. F. T. and Peers, C.: unpublished observations). This discounted any possible effects due to incomplete AII washout before readdition of Ca^{2+} . When extracellular Ca^{2+} was then removed, subsequent applications of carbachol (figs. 3, A and B) or AII (fig. 3C) evoked a second rise of $[Ca^{2+}]_i$. These findings suggest that application of Ca^{2+} to cells after agonist exposure allowed refilling of an intracellular Ca^{2+} store or stores (i.e., capacitative Ca^{2+} entry), which could then be discharged to release Ca^{2+} when agonist was applied for a second time. To investigate this possibility further we examined the actions of the Ca^{2+} -ATPase inhibitor thapsigargin (Thastrup *et al.*, 1990) to modulate the responses illustrated in figure 3. Application of thapsigargin (1 μ M) to cells bathed in Ca^{2+} -free media caused a slowly developing, small increase in $[Ca^{2+}]_i$ (mean 75 ± 4 nM, $n = 5$, e.g., fig. 4A) suggesting that leak of Ca^{2+} from these internal stores was slow in SH-SY5Y cells. To determine the length of time for which cells required exposure to thapsigargin to discharge all their stored Ca^{2+} , we examined (in Ca^{2+} -free solutions) the ability of muscarine (at a supramaximal concentration of 100 μ M) to raise $[Ca^{2+}]_i$ at various times after the onset of thapsigargin application. Figure 4B shows that responses to 100 μ M muscarine declined only over several minutes of thapsigargin exposure, and more than 10 min of thapsigargin treatment were required before responses to muscarinic receptor activation were abolished (i.e., before intracellular Ca^{2+} stores were fully depleted).

Because intracellular Ca^{2+} stores depleted only slowly in the presence of thapsigargin (fig. 4B), but could be discharged rapidly by AII or carbachol when applied in Ca^{2+} -free solutions (fig. 2), we adopted the approach of recording $[Ca^{2+}]_i$ in SH-SY5Y cells while continually perfusing with Ca^{2+} -free solutions containing 1 μ M thapsigargin. Then,

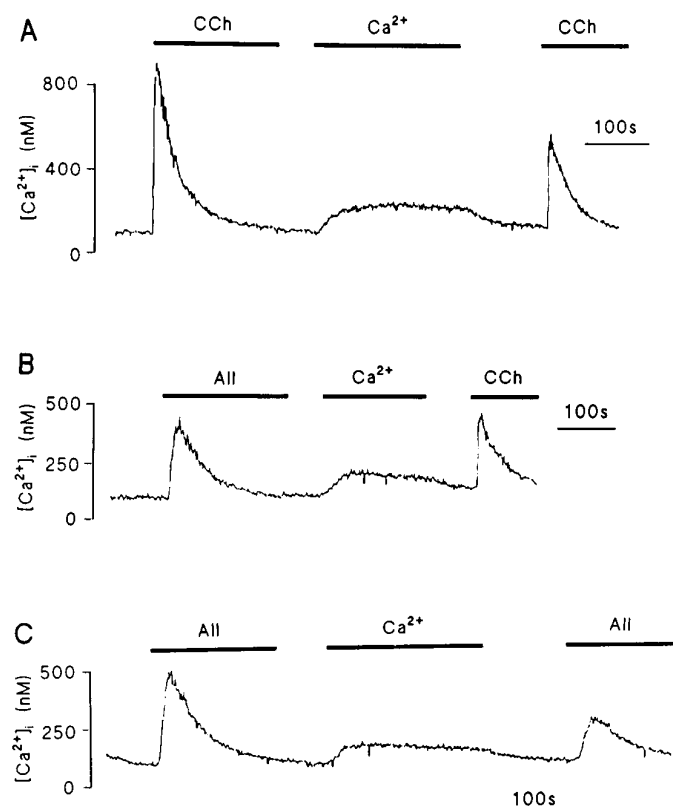


Fig. 3. In all traces (A–C) recordings of $[Ca^{2+}]_i$ commence in solutions in which 2.5 mM Ca^{2+} has been replaced by 1 mM EGTA. Periods of exposure to agonists or to solutions in which 2.5 mM Ca^{2+} replaced EGTA are indicated by solid bars. (A) Carbachol (CCh; 100 μ M) was first applied and after its removal, 2.5 mM Ca^{2+} was added to the perfusate (no EGTA). After removal of Ca^{2+} , 100 μ M carbachol was reapplied. In Ca^{2+} -free solution elicits a second transient response. (B) as in (A), except that 10 nM were first applied to cells instead of CCh. (C) As for (A), except that 10 nM AII was applied instead of CCh before and after application of 2.5 mM Ca^{2+} in the perfusate.

stores could be rapidly and irreversibly emptied by agonist application. Thus, in the continued presence of 1 μ M thapsigargin and after AII application then removal in Ca^{2+} -free solutions, exposure to 2.5 mM Ca^{2+} caused a substantial rise in $[Ca^{2+}]_i$, of 153 ± 16 nM ($n = 14$, e.g., fig. 4C). Similarly, after carbachol application in Ca^{2+} -free solutions containing 1 μ M thapsigargin, exposure to 2.5 mM Ca^{2+} evoked a rise of 173 ± 21 nM ($n = 8$, McDonald, R. L., Vaughan, P. F. T. and Peers, C, unpublished observations). In the continued presence of thapsigargin, a second application of AII (or of carbachol) failed to raise $[Ca^{2+}]_i$ after removal of external Ca^{2+} (e.g., fig. 4C, representative of seven recordings for 10 nM AII and four for 100 μ M carbachol).

Ca^{2+} influx pathways activated by store depletion have been described in a variety of other cell types, and are known to be sensitive to blockade by inorganic ions (Fasolato *et al.*, 1994). We found this pathway in SH-SY5Y cells to be sensitive to blockade by Ni^{2+} : in six recordings, in the presence of thapsigargin and after application then removal of 10 nM AII, exposure of cells to 2.5 mM Ca^{2+} caused a rise in $[Ca^{2+}]_i$ of 135 ± 16 nM. When Ni^{2+} was applied to cells during the Ca^{2+} exposure, the rise of $[Ca^{2+}]_i$ was reversibly reduced by 89 ± 13 nM ($P < .001$, paired *t* test), corresponding to a $67 \pm 9\%$ inhibition of the influx pathway activated by store depletion. Figure 4D shows an example of this effect.

In various other cell types, agonists have been shown to activate Ca^{2+} entry pathways not only by a capacitative (store-depletion mediated) mechanism, but also by an ill-defined pathway that requires receptor occupancy by the agonist (Fasolato *et al.*, 1994; Hughes and Schachter, 1994; Kaplan *et al.*, 1994). We tested for this pathway using procedures indicated in figure 5: in the presence of 1 μ M thapsigargin throughout to prevent store refilling, intracellular stores were discharged in Ca^{2+} -free solutions by the addition of either 10 nM AII (fig. 5A) or 100 μ M carbachol (fig. 5B). Cells were then exposed to 2.5 mM Ca^{2+} and, during the period of raised $[Ca^{2+}]_i$ due to capacitative Ca^{2+} entry, either AII (fig. 5A) or carbachol (fig. 5B) were reapplied. When AII was reapplied, there was no further increase in $[Ca^{2+}]_i$ above that caused by exposure to 2.5 mM Ca^{2+} (e.g., fig. 5A), regardless of whether stores were initially discharged by AII ($n = 3$) or by carbachol ($n = 4$, McDonald, R. L., Vaughan, P. F. T. and Peers, C, unpublished observations). In contrast to this lack of effect of AII, when carbachol was reapplied during exposure to 2.5 mM Ca^{2+} , a further rise of $[Ca^{2+}]_i$ was observed (e.g., fig. 5B; mean rise 90 ± 8 nM, $n = 11$).

In the presence of extracellular Ca^{2+} , carbachol could stimulate Ca^{2+} entry via activation of nicotinic receptors that are present in these cells (Forsythe *et al.*, 1992; Gould *et al.*, 1992; Vaughan *et al.*, 1993; Connor and Henderson, 1996). To investigate this, we examined the effects of DMPP, a selective nicotinic agonist, mecamylamine as a nicotinic antagonist and the muscarinic agonist muscarine. These studies were carried out in both transfected and nontransfected cells with similar results so data have been pooled, and in all experiments 1 μ M thapsigargin was present and stores were depleted with carbachol as in figure 5B. First, in the presence of 1 μ M mecamylamine (which fully blocks nicotinic-induced rises of $[Ca^{2+}]_i$ (Vaughan *et al.*, 1993), 100 μ M carbachol evoked a transient rise of $[Ca^{2+}]_i$ above capacitative entry (e.g., fig. 5C) of 22 ± 8 nM ($n = 5$). Second, muscarine (100 μ M) under the same conditions but in the absence of mecamylamine also evoked a transient rise of 22 ± 2 nM ($n = 12$, e.g., fig. 5D). In the presence of 1 μ M mecamylamine, muscarine produced a similar rise (26 ± 4 nM, $n = 11$). Application of 20 μ M DMPP (a maximal concentration, see Vaughan *et al.*, 1993) elevated $[Ca^{2+}]_i$ above capacitative entry by 37 ± 7 nM ($n = 10$, not shown) and combined application of muscarine and DMPP evoked a rise of 62 ± 12 nM ($n = 12$), which was not significantly different from the effects of carbachol (see above). Thus although part of the additional rise of $[Ca^{2+}]_i$ seen above capacitative entry in response to carbachol is attributable to activation of nicotinic receptors, a significant portion is due to the presence of agonist acting at muscarinic receptors.

Discussion

Of the many receptors coupled to second messenger pathways in SH-SY5Y cells (Vaughan *et al.*, 1995b) the best described is the muscarinic M_3 receptor. Agonists acting at M_3 receptors stimulate production of inositol phosphates, notably IP_3 and IP_4 , which are involved in the release of Ca^{2+} from intracellular stores and the resultant rise of $[Ca^{2+}]_i$ can be observed in ratiometric fluorescence studies as a rapid, transient rise of $[Ca^{2+}]_i$ (Lambert and Nahorski,

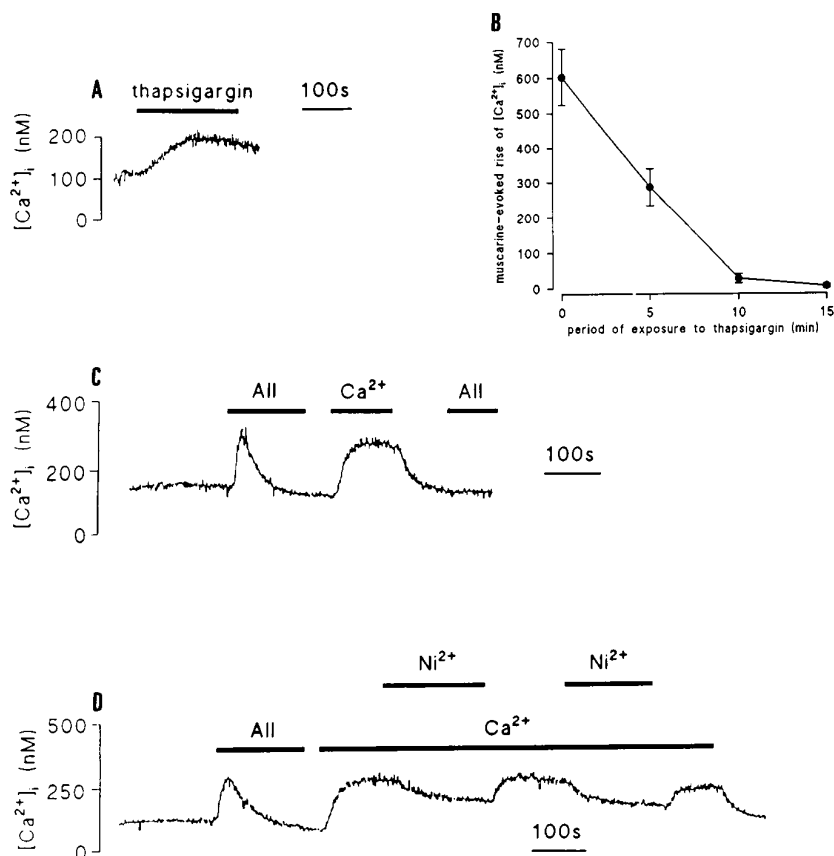


Fig. 4. In (A), (C) and (D) periods of applications of agonists, Ca^{++} or Ni^{++} are indicated by solid bars. (A) Representative example of the effects of $1 \mu M$ thapsigargin on $[Ca^{++}]_i$ in cells perfused with Ca^{++} -free solution (containing $1 mM$ EGTA). (B) Plot of mean (\pm S.E.M., $n = 3-5$ experiments in each case) peak response to $100 \mu M$ carbachol evoked in cells perfused with Ca^{++} -free solutions after varying periods of exposure to $1 \mu M$ thapsigargin. (C) Effects of application of $10 nM$ AII (Ca^{++} -free solution containing $1 mM$ EGTA), followed by addition of $2.5 mM$ Ca^{++} (no EGTA) then, after removal of Ca^{++} , $10 nM$ AII was reapplied. One μM thapsigargin was present throughout, and its application was commenced $2 min$ before the start of the recording. (D) as for (C), $1 \mu M$ thapsigargin was present throughout, and for $2 min$ before commencing recording. Ten nM AII were first applied to cells in Ca^{++} -free perfusate ($1 mM$ EGTA). During addition of $2.5 mM$ Ca^{++} to the perfusate, $1 mM$ Ni^{++} was also applied for the periods indicated.

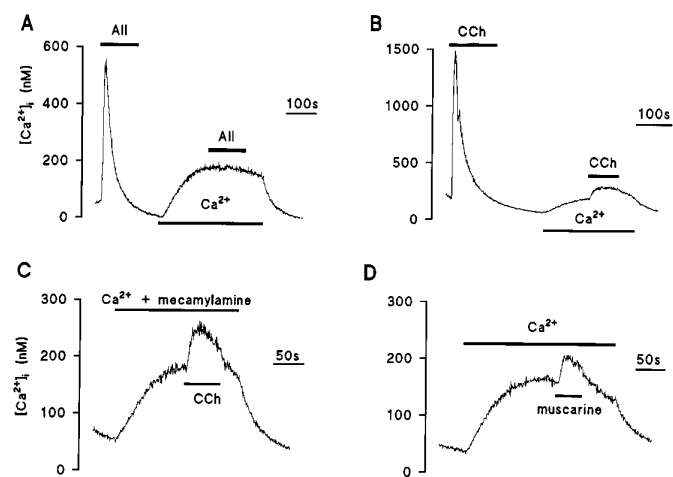


Fig. 5. In all traces (A-D) recordings are commenced in solutions in which Ca^{++} has been replaced by $1 mM$ EGTA, and periods of exposure to agonists or to solutions in which $2.5 mM$ Ca^{++} replaced EGTA are indicated by solid bars. Thapsigargin ($1 \mu M$) was also present throughout and was present for $2 min$ before commencing recordings. (A) After application then removal of AII ($10 nM$), cells were exposed to $2.5 mM$ Ca^{++} and AII was reapplied during exposure to Ca^{++} . (B) as for (A), except that CCh ($100 \mu M$) was applied rather than AII. (C) Effects of readdition of extracellular Ca^{++} together with $1 \mu M$ mecamlamine (after initial discharge of stores with $100 \mu M$ carbachol in Ca^{++} -free perfusate, not shown). During exposure to Ca^{++} and mecamlamine, $100 \mu M$ carbachol were briefly applied. $[Ca^{++}]_i$. (D) as in (C), stores were initially discharged with CCh (not shown), then $2.5 mM$ Ca^{++} reapplied. One hundred μM muscarine were then briefly applied during Ca^{++} exposure.

1990; Lambert *et al.*, 1990; Murphy *et al.*, 1991b; Purkiss *et al.*, 1995). In addition to raising $[Ca^{++}]_i$ via mobilization from such stores, M_3 receptor activation also stimulates Ca^{++} influx across the plasma membrane, giving rise to a sustained elevation of $[Ca^{++}]_i$ although agonist is still present (Lambert and Nahorski, 1990; Murphy *et al.*, 1991b; Lambert *et al.*, 1990; Grudt *et al.*, 1996; Connor and Henderson, 1996). The mechanism underlying M_3 receptor-mediated Ca^{++} influx has not been fully characterized, but is not thought to involve voltage-gated Ca^{++} channels, a pertussis toxin-sensitive G-protein operated Ca^{++} channel or an IP_3 or IP_4 -operated Ca^{++} channel (Lambert and Nahorski, 1992). Instead, M_3 -mediated Ca^{++} entry is believed to involve a receptor-operated Ca^{++} channel and/or capacitive refilling of discharged intracellular Ca^{++} stores (Lambert and Nahorski, 1990; Lambert *et al.*, 1990). We have compared the actions of AII with those of carbachol, to characterise the regulation of $[Ca^{++}]_i$ by activation of recombinant rat AT_{1A} receptors and also to investigate further the Ca^{++} influx pathways mediated by M_3 receptors.

Our study demonstrates that recombinant rat AT_{1A} receptors in human neuroblastoma (SH-SY5Y) cells are coupled to the regulation of $[Ca^{++}]_i$. The lack of effect of AII on untransfected cells suggests that SH-SY5Y do not express native AII receptors, or that they are nonfunctional, or not coupled to mobilization of $[Ca^{++}]_i$. Our binding studies indicate that the first of these possibilities is most likely. In transfected cells, therefore, responses to AII must be mediated by AT_{1A} receptors and this is supported by the fact that losartan fully blocked the effects of AII (McDonald *et al.*, 1995a).

Prolonged exposure to AII revealed a biphasic response,

with an initial rapid but transient rise of $[Ca^{++}]_i$ which declined to an elevated plateau level (fig. 1C). The initial transient rise is attributable to Ca^{++} mobilization from intracellular store(s) because it could be observed in Ca^{++} -free solutions, and this effect is most likely to be mediated by the generation of IP_3 . Stimulation of AT_1 receptors is known to activate phospholipase C and stimulate IP_3 production in various cell types (Timmermans *et al.*, 1993). In SH-SY5Y cells, activation of M_3 receptors also stimulates IP_3 generation (see above), and we have demonstrated that, in Ca^{++} -free solutions, prior administration of carbachol blocks the effects of AII and vice versa (fig. 2). These findings suggest that rat AT_{1A} receptors expressed in SH-SY5Y cells couple to the generation of IP_3 and mobilize the same pool of Ca^{++} that is released by activation of M_3 receptors via IP_3 generation.

The plateau phase of elevated $[Ca^{++}]_i$ in response to AII application is attributable to Ca^{++} entry across the plasma membrane, because it was not observed in Ca^{++} -free solutions (fig. 2). This Ca^{++} entry was not via L-type voltage-gated Ca^{++} channels, since it was unaffected by a supra-maximal concentration of nifedipine. SH-SY5Y cells possess both L- and N-type Ca^{++} channels (*e.g.*, Reeve *et al.*, 1994, 1995). We have not examined whether AII-induced Ca^{++} entry is mediated by N-type Ca^{++} channels, because this possibility is considered highly unlikely: N-type Ca^{++} channels contribute less than L-type to K^+ -evoked NA release, and indeed only contribute at all when external $[K^+] \geq 60$ mM (McDonald *et al.*, 1994b; also Vaughan, P. F. T., unpublished observations). Furthermore, although muscarinic agonists can generate inward currents (Forsythe *et al.*, 1992) and depolarize SH-SY5Y cells (Akerman, 1989), Ca^{++} entry stimulated by activation of M_3 receptors in SH-SY5Y cells is insensitive to blockers of L- and N-type Ca^{++} channels (Lambert *et al.*, 1990). In addition, muscarine acting at M_1 receptors inhibits L- and N-type Ca^{++} channels (McDonald *et al.*, 1994b), and via M_3 receptors inhibits N-type channels in these cells (Reeve *et al.*, 1995).

In a variety of cell types, discharge of intracellular Ca^{++} stores can in itself stimulate Ca^{++} influx via a mechanism that does not require receptor occupancy by agonist (Fasolato *et al.*, 1994). This capacitative Ca^{++} entry can be observed in experiments initially using Ca^{++} -free media where, after an initial transient rise of $[Ca^{++}]_i$ in response to agonist, application of extracellular Ca^{++} causes a rise of $[Ca^{++}]_i$ in the absence of agonist. We have shown this to occur in SH-SY5Y cells after activation of muscarinic receptors (fig. 3). Our study indicates that this capacitative Ca^{++} entry pathway is also activated after stimulation of recombinant AT_{1A} receptors (fig. 3 and 4). This Ca^{++} entry refills Ca^{++} stores in SH-SY5Y cells, because second responses to AII can be observed in Ca^{++} -free solutions if Ca^{++} is applied to cells between agonist applications (fig. 3). If it is not, no second response to AII (or carbachol) is observed (fig. 2).

Capacitative Ca^{++} entry was further examined with the use of thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{++} ATPase that serves to pump Ca^{++} from the cell cytosol into intracellular stores (Thastrup *et al.*, 1990). Thapsigargin alone caused small, slowly developing rises of $[Ca^{++}]_i$ and in its continued presence, after agonist application in Ca^{++} -free solution, exposure to external Ca^{++} caused rises of $[Ca^{++}]_i$ similar to those seen in the absence of thapsigargin. However, second applications of agonist now failed

to evoke rises of $[Ca^{++}]_i$, indicating that the influxed Ca^{++} is required to be pumped into the endoplasmic reticulum via a Ca^{++} ATPase to be discharged when AII or carbachol is reapplied (fig. 4). Capacitative Ca^{++} entry pathways in other cell types have not been thoroughly characterized to date, but have been shown to be sensitive to inorganic cations (Fasolato *et al.*, 1994). We show that this entry pathway can be strongly inhibited by 1 mM Ni^{++} (fig. 4D), which therefore may serve as a useful tool in investigating the physiological relevance of this Ca^{++} entry pathway to neuronal functioning.

Results illustrated in figure 5 indicate a clear difference in Ca^{++} entry pathways activated by AII and carbachol. In the case of AII, no further increases in $[Ca^{++}]_i$ were observed during store-depletion activated entry (fig. 5A), suggesting that this is the only pathway activated by stimulation of recombinant rat AT_{1A} receptors. In sharp contrast, carbachol stimulated a further rise of $[Ca^{++}]_i$ during store-depletion activated Ca^{++} entry (fig. 5B) that was partly attributable to influx via nicotinic receptors and partly due to muscarinic receptors (fig. 5, C and D). This additional rise cannot be attributed to release from an internal store, as these were previously discharged and recordings were made in the presence of thapsigargin to prevent any refilling. Thus, this additional rise of $[Ca^{++}]_i$ must be attributed to activation of an additional influx pathway caused by muscarinic receptor occupancy. Previous studies on muscarinic receptor activation of Ca^{++} influx in SH-SY5Y have not distinguished between store-depletion activated and receptor-mediated entry pathways (Lambert and Nahorski, 1992), and have not discounted the possibility of both existing in these cells. Our results provide evidence for the presence of both in SH-SY5Y cells. In a most recent, comparable study, Grudt *et al.*, (1996) indicated that Ca^{++} entry (above capacitative influx) attributable to muscarinic receptor occupancy was barely detectable in SH-SY5Y cells. However, these workers applied a muscarinic agonist before readdition of Ca^{++}_o as well as during Ca^{++} exposure, and compared results with those obtained on simply reading Ca^{++}_o . Our results (fig. 5, C and D) indicate that this would lead to severe underestimation of receptor mediated Ca^{++} influx because responses to agonists during capacitative Ca^{++} entry were transient in nature. Thus, our study provides the first compelling evidence for a Ca^{++} entry pathway activated by muscarinic receptor occupancy.

In summary, our results demonstrate that recombinant AT_{1A} receptors expressed in SH-SY5Y cells can couple to the same, most likely IP_3 -mediated release of Ca^{++} from intracellular stores as is activated by carbachol via muscarinic receptors. Furthermore, AT_{1A} receptor activation can stimulate capacitative Ca^{++} influx that is induced by store depletion and can be observed in the absence of agonist. We have also demonstrated that carbachol stimulates three Ca^{++} entry pathways, including the capacitative pathway that is indistinguishable from that activated by AT_{1A} receptors. In addition two pathways dependent on the presence of agonist acting at nicotinic and muscarinic receptors have been demonstrated. It is likely that this additional muscarinic pathway accounts for the fact that carbachol is a more effective secretagogue than AII in these cells (see above). The responses of $[Ca^{++}]_i$ to activation of AT_{1A} receptors may well be involved in the mechanisms underlying the facilitation of NA

release caused by AII acting at sympathetic ganglia and at postganglionic sympathetic nerve endings (Reid, 1992).

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