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Noradrenaline synthesis after sympathetic nerve activation in rat atria and its dependence on calcium but not CAM kinase II and protein kinases A or C

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1 The biosynthesis of noradrenaline following sympathetic nerve activation was investigated in rat atria. In particular the time course of noradrenaline synthesis changes, the relationship of changes in synthesis to transmitter release and the possible roles of second messengers and protein kinases were examined.

2 Rat atria incubated with the precursor [^{3}H]-tyrosine synthesized [^{3}H]-noradrenaline. Synthesis was enhanced following pulsatile electrical field stimulation (3 Hz for 5 min) with the bulk of the increase occurring in the first 45 min after the commencement of electrical stimulation. In separate experiments rat atria were pre-incubated with [^{3}H]-noradrenaline and the radioactive outflow in response to electrical field stimulation (3 Hz for 5 min) was taken as an index of noradrenaline release.

3 Stimulation-induced (S-I) noradrenaline synthesis was significantly correlated to S-I noradrenaline release for a variety of procedures which modulate noradrenaline release by mechanisms altering Ca^{2+} entry into the neurone ($r^2=0.99$): those which decreased release: tetrodotoxin (0.3 μM), Ca^{2+}-free medium, lowering the frequency of nerve activation to 1 Hz, and those which increased release, tetraethylammonium (0.3 mM), phentolamine (1 μM) and the combination of phentolamine (1 μM) and adenosine (10 μM). On the strength of this relationship we suggest that Ca^{2+} entry is a determining factor in S-I synthesis changes rather than the amount of noradrenaline released. Indeed the reduction in noradrenaline release with the calmodulin-dependent protein (CAM) kinase II inhibitor KN-62 (10 μM) which acts subsequent to Ca^{2+} entry, did not affect S-I synthesis.

4 The cell permeable cyclic AMP analogue, 8-bromoadenosine 3′, 5′-monophosphate (BrcAMP, 90 and 270 μM), dose-dependently increased basal [^{3}H]-noradrenaline synthesis in unstimulated rat atria. This effect was antagonized by the selective protein kinase A (PKA) antagonist, Rp-8-chloroadenosine 3′, 5′-cyclic monophosphorothioate (RClcAMPS, 300 μM), suggesting that PKA activation enhances basal noradrenaline biosynthesis in sympathetic nerve terminals.

5 The protein kinase inhibitors, KN-62 (CAM kinase II, 10 μM), RClcAMPS (PKA, 300 μM), polymyxin B (protein kinase C (PKC), 21 μM) and staurosporine (PKC, PKA and CAM kinase II, 0.1 μM) did not affect S-I synthesis, although KN-62, polymyxin B and staurosporine decreased S-I release. We conclude that S-I synthesis is triggered by Ca^{2+} entering the neurone but that the signalling pathway does not involve classical protein kinases and appears distinct from the steps involved in transmitter release.

Keywords: Sympathetic nerves; noradrenaline synthesis; calcium; protein kinases; tyrosine hydroxylase

Introduction

In catecholaminergic neurones there is an increase in catecholamine biosynthesis following catecholamine release to replenish the transmitter lost during the release process (see Zigmond *et al.*, 1989). This is due to enhanced catalytic activity of tyrosine hydroxylase (EC; tyrosine 3-mono-oxygenase, EC 1.14.16.2), the rate limiting enzyme in the biosynthetic pathway of catecholamines (see Zigmond *et al.*, 1989). Tyrosine hydroxylase is a substrate for various protein kinases including protein kinase A (Chalfie *et al.*, 1979), CAM kinase II (Yamauchi & Fujisawa, 1981), protein kinase C (Albert *et al.*, 1984) and MAP kinase I and II (Haycock *et al.*, 1992; Haycock, 1993) and in general phosphorylation by each of these kinases independently can lead to an increase in the catalytic activity of tyrosine hydroxylase (see Tachikawa *et al.*, 1986; 1987; Yanagihara *et al.*, 1986; Haycock, 1993). Furthermore, *in situ* there appears to be multiple sites of phosphorylation following nerve or cell depolarization (e.g. Haycock & Wakade, 1992) consistent with these protein kinases being physiologically important.

A working hypothesis is that during nerve depolarization there is an increase in second messengers which activates the various kinases to enhance transmitter synthesis (Haycock & Haycock, 1991). A difficulty with this hypothesis is that increases in tyrosine hydroxylase activity and/or phosphorylation in intact neural systems have only been observed after pharmacological depolarization with K^+ (El Mestikawy *et al.*, 1983; Rittenhouse & Zigmond, 1991), veratridine (Horwitz & Perlman, 1984), the Ca^{2+} ionophore, A23187 (Birch & Fillenz, 1985), or with nerve stimulation of supraphysiological intensity: rat striatum 30 Hz for 20 min (Haycock & Haycock, 1991). Consequently, it is unclear whether the results of these studies are relevant to the physiological situation where transmitter is released in smaller amounts by low frequency nerve stimulation and where the demands on the synthetic machinery would be far less.

Previous workers have established that postganglionic sympathetic neurones respond to nerve activation with increases in tyrosine hydroxylase activity (Morgenroth *et al.*, 1975; Weiner *et al.*, 1978) and noradrenaline biosynthesis (Cloutier & Weiner, 1973) albeit with high stimulation parameters e.g. 10–20 Hz for up to 60 min. Our present study was designed to study noradrenaline biosynthesis in the sympathetic nerves of rat atria in response to sympathetic nerve activation in the physiological range (1–3 Hz) over a short

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duration (5 min). This is within the firing rates seen in single fibre recordings (Sato *et al.*, 1986; Sato & Schaible, 1987). In particular we wished to investigate the time course of noradrenaline synthesis changes, the relationship of changes in synthesis to transmitter release and the possible role of second messengers and protein kinases in elevating synthesis after nerve activation. We adopted a pharmacological approach using activators and inhibitors of second messenger pathways to elucidate their importance.

Methods

[^{3}H]-noradrenaline synthesis from [^{3}H]-tyrosine

Male Sprague-Dawley rats (130–190 g) were decapitated and the atria were dissected free and attached to a stainless steel tissue hook, between two platinum electrodes, in a jacketed organ bath containing 1.0 ml physiological salt solution (PSS). The PSS also contained 30 μM L-tyrosine which is in the range of the plasma concentration of endogenous tyrosine found in conscious male rats as measured in our laboratory with high-performance liquid chromatography (h.p.l.c.) coupled with electrochemical detection (unpublished observations). The deduced Michaelis-Menton constant (K_m) for tyrosine was found to be 60 μM from a Line-Weaver Burke plot (see Cloutier & Weiner, 1973). The PSS was maintained at 37°C and continuously gassed with a mixture of 95% O_2 and 5% CO_2. A 30 min equilibration period was allowed with the bathing solution being replaced every 3–5 min. For determination of [^{3}H]-noradrenaline synthesis, atria were incubated in L-[2, 6-^{3}H]-tyrosine (final bath concentration total of 31 μM, 37 μCi ml^{-1}) for 45 min without changing the bathing solution. When drugs were used they were present from 10–30 min before the addition of [^{3}H]-tyrosine (10 min before: tetrodotoxin, phentolamine, clonidine, adenosine and the combination of 8-bromoadenosine 3′, 5′-cyclic monophosphate (BrcyclicAMP) and 3-isobutyl-1-methyl-xanthine (IBMX); 15 min before: tetraethylammonium, staurosporine and phorbol 12, 13-dibutyrate; 20 min before: KN-62; 30 min before: polymyxin B) and remained until the end of the experiment. In some experiments atria were electrically stimulated (14 V, 1 or 3 Hz, square wave pulses of 1 ms pulse duration for 5 min) immediately after the addition of [^{3}H]-tyrosine except for some of the data in Figure 1 (see Figure 1 legend for details). Forty five min after the addition of [^{3}H]-tyrosine, the atria were blotted dry and placed in 1.0 ml $HCLO_4$ (0.3 M). The atria were then homogenized and the homogenate was stored at −40°C for up to 3 days.

The amount of [^{3}H]-noradrenaline synthesized from [^{3}H]-tyrosine in atria was measured by h.p.l.c. of 200 μl supernatant derived after centrifugation of the tissue homogenate at 14,000 *g* for 15 min at 4°C. A reverse phase column (EXCIL C18 5M-Alt 230, Activon, Australia) was used. The mobile phase consisted of Na_2HPO_4 (60 mM, pH 3.1) containing heptane sulphonic acid (5 mM), Na_2EDTA (0.5 mM) and 25 ml l^{-1} methanol. The column eluate was collected in a fraction collector and the radioactivity in each fraction quantified by liquid scintillation counting with 2.0 ml Picofluor 40 (Packard Instruments, Melbourne, Australia). Corrections were made for counting efficiency by use of an automatic external standard and results are expressed as disintegrations per min (d min^{-1}). The noradrenaline fraction was identified based on the retention time of endogenous noradrenaline which was distinct from other possible metabolites. The retention times were: 3, 4-dihydroxyphenylglycol (4.5 min), noradrenaline (12.8 min), L-DOPA (14.5 min), tyrosine (18.9 min) and dopamine (66.5 min) as measured by electrochemical detection on an ESA Coulochem detector (ESA, Bedford, U.S.A.) with a two stage detector (#5011). For all compounds, except tyrosine, the oxidation voltage was +0.35 V and for tyrosine it was +0.65 V, with the screen detector being set at +0.05 V and +0.5 V, respectively. The recovery of the [^{3}H]-noradrenaline fraction was greater than 95% as measured with [^{3}H]-noradrenaline standards. [^{3}H]-tyrosine standards showed no cross contamination of the noradrenaline fraction.

Calculation of synthesis

Basal synthesis was calculated as the amount of [^{3}H]-noradrenaline synthesized after 45 min contact with [^{3}H]-tyrosine in the absence of electrical stimulation. In stimulation experiments the [^{3}H]-noradrenaline synthesized in the 45 min period following the commencement of electrical stimulation was measured. The stimulation-induced synthesis was calculated as the mean [^{3}H]-noradrenaline synthesis formed in stimulation experiments minus the respective mean basal formation. All results were normalized such that control = 100.

[^{3}H]-noradrenaline synthesis from [^{3}H]-tyrosine-effect of RClcAMPS

The experimental protocol was altered to minimize the amount of Rp-8-chloradenosine 3′, 5′-cyclic monophosphorothioate (RClcAMPS) used. In these experiments the right atrium alone was used and attached to a stainless steel hook in a jacketed bath (as above) containing only 0.7 ml PSS. A 10 min equilibration period was allowed with the bathing solution being replaced every 2–3 min. The atrium was incubated with RClcAMPS for a total of 90 min (t = 0 to t = 90) without the bathing solution being replaced. At t = 45 L-[2, 6-^{3}H]-tyrosine was then added to the bathing solution (final bath concentration total of 31 μM, 37 μCi ml^{-1}) and at t = 90 the formation of [^{3}H]-noradrenaline was determined as described above. In some experiments electrical stimulation (3 Hz for 5 min) was applied at t = 45.

[^{3}H]-noradrenaline release experiments

Atria were dissected free and placed in 1.0 ml PSS (as described above). A 25–30 min equilibration period was allowed with the bathing solution replaced every 3–5 min. The atria were then incubated in (−)-[ring 2, 5, 6-^{3}H]-noradrenaline (0.2 μM, 10 μCi ml^{-1}) for 20 min. After this incubation period the atria were washed every min for 10 min and then every 2 min for 50 min with fresh PSS. Following this washing period the bathing solution was collected every 5 min for 90 min during which time there were two periods of electrical stimulation of the atria (14 V, 3 Hz, square wave pulses of 1 ms pulse duration for 5 min) at 15 min and 60 min after the onset of collecting. Drugs under investigation were added from various times before the second period of electrical stimulation and were present in the bathing solution until the completion of the experiment (10 min before: tetrodotoxin, phentolamine, adenosine and the combination of BrcyclicAMP and IBMX; 15 min before: tetraethylammonium, staurosporine and phorbol 12, 13-dibutyrate; 20 min before: KN-62; 30 min before: polymyxin B). At the end of the collection period the atria were dissolved in 2.0 ml Soluene 350 (Packard Instruments, Melbourne, Australia). The amount of radioactivity in the tissue samples and the bathing solution was determined by adding 6.0 ml and 2.0 ml Picofluor-40 to each sample, respectively, followed by liquid scintillation counting. Corrections were made for counting efficiency by an automatic external standard and results are expressed as disintegrations per min (d min^{-1}).

[^{3}H]-noradrenaline release experiments–effect of RClcAMPS

In these experiments the right atrium alone was used and it was placed in only 0.7 ml PSS. There were small protocol changes from above in that there were two periods of

electrical stimulation (14 V, 3 Hz, square wave pulses of 1 ms pulse duration for 5 min at $t=15$ and $t=90$ min). RClcAMPS was added to the bathing solution at $t=45$ where it remained until $t=85$. The bathing solution was then replaced with fresh PSS containing RClcAMPS at $t=85$ and $t=90$. Control experiments were performed with RClcAMPS vehicle (H_2O) in PSS.

Calculation of results

The basal (non-stimulated spontaneous) outflow of radioactivity from atria was taken as the mean radioactive content of the bathing solution during the 5 min period immediately before electrical stimulation and the 5 min period collected 20 min after the onset of electrical stimulation. The stimulation-induced (S-I) outflow of radioactivity was calculated by subtracting the total amount of radioactivity in the calculated basal outflow from the total amount of radioactivity in the three 5 min samples collected during and immediately after electrical stimulation. In all cases the S-I outflow of radioactivity and basal outflow of radioactivity were expressed as a fraction of the total tissue content of radioactivity at the time of stimulation (fractional outflow, FR). The fractional basal and the S-I outflow of radioactivity in the second stimulation period is expressed as a percentage of that in the first stimulation period. All results were normalized such that 3 Hz control = 100.

Measurement of endogenous noradrenaline release from rat atria during electrical stimulation

Atria were dissected and prepared as described above. A 10 min equilibration period was allowed with the bathing solution being replaced every 2–3 min with 1.0 ml PSS (which also contained 30 μM L-tyrosine) maintained at 37°C and continuously gassed with a mixture of 95% O_2 and 5% CO_2. The bathing solution was then collected every 2 min for 14 min and placed in an ice cold vial containing 1.0 ml HCl (1 M), 100 μl Na_2EDTA (0.27 M) and 100 μl Na_2SO_3 (1 M). After the second collection, the atria were electrically stimulated (14 V, 3 Hz, square wave pulses of 1 ms pulse duration) for 5 min. In those studies where phentolamine was used this drug was added to the bathing solution 10 min before the commencement of electrical stimulation. Vials were then frozen and stored at −40°C.

Within 1 week of sample freezing and storage the sample was allowed to thaw out. After the thawing, 0.8 ml was adjusted to pH 8.3–8.5 with Tris acetate (2 M) buffer pH 8.7 ($\approx$500 μl). The samples were poured onto alumina (200 mg) in a glass column stoppered with a glass fibre filter. The columns were then washed twice with 1.0 ml H_2O. Then 500 μl of $HClO_4$ (0.1 M) was added to the column and the eluate was collected. The amount of noradrenaline in 100 μl of the $HClO_4$ eluate was determined by h.p.l.c. coupled with electrochemical detection. A reverse phase column was used (EXCIL, C18 5M-Alt 230). The mobile phase consisted of Na_2HPO_4 (60 mM) pH 3.1 containing heptane sulphonic acid (2 mM), Na_2EDTA (0.5 mM) and 15 ml l^{-1} methanol. The electrochemical detector was an ESA Coulochem 5100 with an ESA 5011 analytical cell. The first electrode potential was +0.06 V and the detecting electrode was +0.30 V. The mean recovery of noradrenaline by this technique was 89.2±2.7% ($n=12$). Corrections were made for incomplete recoveries. The spontaneous (non-stimulated) outflow of noradrenaline from atria was taken as the mean noradrenaline content of the bathing solution in the 2 min period immediately before (second collection vial) electrical stimulation and in the 2 min period collected 10 min after the commencement after electrical stimulation. The stimulation-induced (S-I) outflow of noradrenaline was calculated by subtracting the total amount of noradrenaline in the calculated spontaneous outflow from the total amount of noradrenaline in the four 2 min samples collected after the commencement of electrical stimulation. All results were normalized such that 3 Hz control = 100.

Physiological salt solution (PSS)

The PSS consisted of (mM): NaCl 117.7, KCl 4.6, $MgSO_4$ 0.8, KH_2PO_4 1.0, D-(+)-glucose 11.1, $NaHCO_3$ 25.0, $CaCl_2$ 1.8, Na_2EDTA 0.067 and L-ascorbic acid 0.14. In those experiments where endogenous noradrenaline release was determined and [^{3}H]-noradrenaline synthesis was measured from [^{3}H]-tyrosine, the PSS also contained 30 μM L-tyrosine.

Radiochemicals and drugs

The following radiochemicals and drugs were used: L-[2,6-^{3}H]-tyrosine, specific activity 46–51 Ci $mmol^{-1}$ (Amersham, Aylesbury, UK); (−)-[ring 2,5,6-^{3}H]-noradrenaline, specific activity 43.7 Ci $mmol^{-1}$ (Dupont-NEN, Boston, U.S.A.); adenosine, polymyxin B sulphate, tetraethylammonium chloride, tetrodotoxin, clonidine HCl, A23187, L-tyrosine hydrochloride and 3-isobutyl-1-methyl-xanthine (Sigma, St Louis, U.S.A.); phentolamine hydrochloride (Ciba-Geigy, Sydney, Australia); 8-bromoadenosine 3′, 5′-cyclic monophosphate, and staurosporine (Boehringer-Mannheim, Mannheim, Germany); 4β-phorbol 12, 13-dibutyrate (LC Laboratories, Woburn, U.S.A.); Rp-8-chloroadenosine 3′, 5′-cyclic monophosphorothioate (RClcAMPS, Biolog Life Sciences Institute, Bremen, Germany); 1-[N, O-bis (5-isoquinolinesulphonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62, Research Biochemicals International, Natick, U.S.A.). The drugs 3-isobutyl-1-methyl-xanthine, 8-bromoadenosine 3′, 5′-cyclic monophosphate, 4β-phorbol 12, 13-dibutyrate and RClcAMPS were initially dissolved in H_2O before being further diluted in PSS. The drugs staurosporine and KN-62 were initially dissolved in dimethyl sulphoxide before being further diluted in PSS. Control experiments were conducted with the corresponding concentration of dimethyl sulphoxide (up to 0.01% v:v). L-Tyrosine hydrochloride was dissolved after sonication in H_2O for 30 min. All other drugs were dissolved directly into PSS.

Statistical analysis of results

All data are expressed as the mean and standard error of the mean (s.e. mean) with number of observations (n) being given. Data were analysed by two-way analysis of variance (ANOVA) or unpaired two-tailed Student's t tests. In some cases, least squares regression analysis was performed on data selected '*a priori*'. The statistical package SYSTAT was used for all analyses. Probability levels of less than 0.05 ($P<0.05$) were taken to indicate statistical significance in all cases.

Results

[^{3}H]-noradrenaline synthesis-time course

Incubation of rat atria in [^{3}H]-tyrosine for 45 min resulted in the basal formation of [^{3}H]-noradrenaline. This was measured in separate experiments at the end of different time intervals after the dissection of the atria and was 2.9±0.3 pmol per atria ($n=8$) corresponding to the 0–45 min time interval and 3.4±0.5 pmol per atria ($n=3$) corresponding to the 135–180 min time interval. These values were not significantly different ($P>0.05$, Student's t test). Electrical field stimulation (3 Hz for 5 min) of rat atria was performed at $t=0$. As can be seen in Figure 1 the majority of the increase in [^{3}H]-noradrenaline synthesis following the stimulation was observed in the first 45 min. In all subsequent experiments below, stimulation-induced (S-I) noradrenaline synthesis refers to the synthesis in the first

45 min period at the commencement of which the electrical stimulation (3 Hz for 5 min) was applied.

Relationship between the effects of treatments on S-I [^{3}H]-noradrenaline release and [^{3}H]-noradrenaline synthesis

The S-I fractional outflow of radioactivity from rat atria incubated with [^{3}H]-noradrenaline was taken as an index of S-I noradrenaline outflow. In all experiments there were two periods of electrical stimulation with drug or manipulation only present during the second stimulation period. The mean S-I outflow of radioactivity in the first stimulation period was 281529 ± 7312 ($n=78$) d min^{-1} and the outflow returned to basal levels after 10 min. Several procedures were selected to vary the S-I noradrenaline outflow (Figure 2): S-I noradrenaline outflow was eliminated with tetrodotoxin (0.3 μM), and Ca^{2+}-free PSS, it was reduced with a 1 Hz stimulation frequency and was enhanced by each of tetraethylammonium (0.3 mM) and phentolamine (1 μM) and the effect of phentolamine was itself reduced by adding adenosine (10 μM) (Figure 2). These procedures were chosen *a priori* for their diversity, yet similarity in that they modulate noradrenaline release by altering Ca^{2+} entry (see Discussion) and also chosen to produce a graded change in S-I transmitter release. None of these procedures affected the basal outflow of radioactivity (not shown).

In separate experiments the effects of the above procedures and drugs on S-I [^{3}H]-noradrenaline synthesis from [^{3}H]-tyrosine were measured. None of the drugs or procedures affected the basal synthesis of [^{3}H]-noradrenaline (Table 1). The S-I noradrenaline synthesis was eliminated with tetrodotoxin (0.3 μM), and Ca^{2+}-free PSS, it was reduced by a 1 Hz stimulation frequency and was enhanced by each of tetraethylammonium (0.3 mM) and phentolamine (1 μM) and the effect of phentolamine was itself reduced by adding adenosine (10 μM) (Figure 2). Importantly, a linear relationship was observed when the effects of the various treatments on S-I fractional release of radioactivity from rat atria were plotted against the effects of these treatments on S-I [^{3}H]-noradrenaline synthesis from [^{3}H]-tyrosine (Figure 2). To examine whether

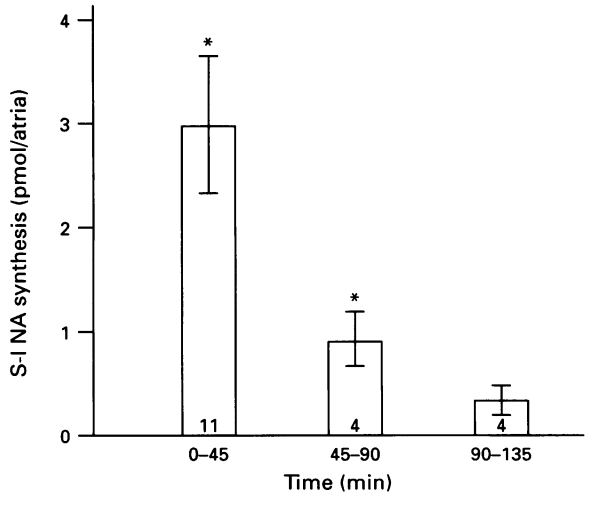

Figure 1 The time course of increases in stimulation-induced (S-I) [^{3}H]-noradrenaline (NA) synthesis from [^{3}H]-tyrosine following field stimulation of rat atria at (time) $t=0$ (3 Hz for 5 min). Means and s.e. mean are shown, and the number of observations (n) is at the base of each column. [^{3}H]-tyrosine was added for 45 min either at $t=0$, $t=45$ or $t=90$ min. The [^{3}H]-noradrenaline formation at the end of each of these times was expressed as S-I noradrenaline synthesis after subtracting the mean basal formation of [^{3}H]-noradrenaline over 45 min. Based on the specific activity of the [^{3}H]-tyrosine in the bathing solution, the results are expressed as pmol noradrenaline synthesized per atria. *Significantly above basal, $P<0.05$, Student's t test with Bonferroni correction. See text for basal synthesis values.

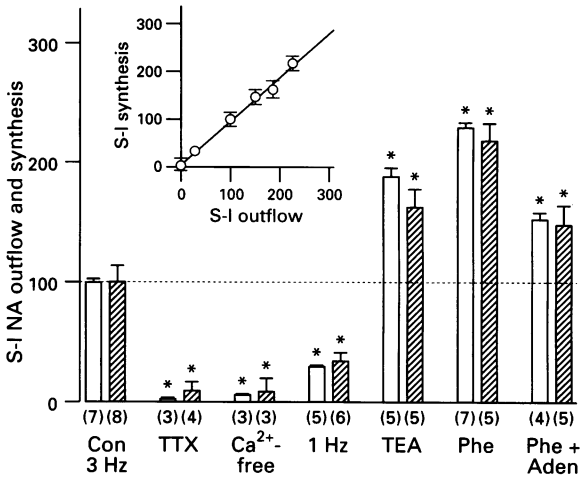

Figure 2 Comparison of stimulation-induced (S-I) [^{3}H]-noradrenaline (NA) outflow (open columns) and S-I [^{3}H]-NA synthesis (hatched columns) after electrical field stimulation of rat isolated atria at 3 Hz for 5 min with treatments which alter Ca^{2+} entry into nerve terminals. The drugs and manipulations used at 3 Hz stimulation were: control (Con), tetrodotoxin (0.3 μM, TTX), the absence of extracellular Ca^{2+} (Ca^{2+}-free), tetraethylammonium (0.3 mM, TEA), phentolamine (1 μM, Phe) and the combination of phentolamine and adenosine (1 μM, Phe + 10 μM, Aden). In addition some atria were stimulated at 1 Hz (for 5 min). The S-I noradrenaline outflow was measured in atria incubated with [^{3}H]-noradrenaline and for 3 Hz control experiments, $FR_2/FR_1 = 0.93 \pm 0.02$ ($n=7$). In separate experiments S-I [^{3}H]-noradrenaline synthesis was measured in atria incubated with [^{3}H]-tyrosine and was found to be 4.26 ± 0.57 pmol/atria ($n=8$) based on the specific activity of the [^{3}H]-tyrosine in the bathing solution. All results were normalized such that 3 Hz control = 100. Means and s.e. mean are shown, and the number of observations (n) is in parentheses at the base of each column. *Significant difference from the respective 3 Hz control experiments, $P<0.05$, Student's t test with Bonferroni correction. Inset: there was a strong linear relationship between the amount of noradrenaline released on stimulation and the amount of noradrenaline synthesized in response to the stimulation: S-I synthesis = 0.9 × S-I release + 6.5; $r^2 = 0.99$, $P<0.001$. See Table 1 for basal synthesis values.

Table 1 Effect of drugs on basal [^{3}H]-noradrenaline synthesis from [^{3}H]-tyrosine in rat isolated atria

	(%) Basal synthesis Mean	s.e.mean	n
Control (Figures 1 and 2)	100.0	10.3	9
Tetrodotoxin (0.3 μM)	104.8	10.8	4
Ca^{2+} free	102.1	8.7	3
Tetraethylammoniun (0.3 mM)	110.6	8.6	4
Phentolamine (1 μM)	97.2	9.6	5
Phentolamine (1 μM) + adenosine (10 μM)	105.2	10.8	5
Control (Figure 4)	100.0	9.7	11
KN-62 (10 μM)	84.8	16.8	7
BrcyclicAMP (90 μM) + IBMX (100 μM)	168.3*	27.3	9
Polymyxin B (21 μM)	100.1	10.5	5
Staurosporine (0.1 μM)	108.4	5.8	5
Phorbol 12,13-dibutyrate (0.1 μM)	120.5	11.2	5
Control	100.0	11.9	12
Clonidine (0.1 μM)	115.3	9.0	6

Tissues were incubated with [^{3}H]-tyrosine in the presence or absence of the above drugs and the formation of [^{3}H]-noradrenaline was measured 45 min later by h.p.l.c. Drugs under investigation were added from various time points before the addition of [^{3}H]-tyrosine (see Methods) and all results were normalized such that control = 100%. *Significantly different from control ($P<0.05$, Student's t test).

the relationship could be explained by direct activation of presynaptic α-adrenoceptors modulating synthesis, the actions of clonidine (0.1 μM) on basal synthesis were examined and it was found to have no effect (Table 1).

Relationship between endogenous S-I noradrenaline outflow and [^{3}H]-noradrenaline synthesis

The amount of endogenous S-I noradrenaline outflow from rat atria was measured for 3 treatments: 1 Hz stimulation, 3 Hz stimulation and 3 Hz stimulation in the presence of phentolamine (1 μM). This produced a graded change in noradrenaline outflow. This was then plotted against the corresponding measured levels of [^{3}H]-noradrenaline synthesis (Figure 3). Regression analysis of all treatments revealed a linear relationship (Figure 3).

Effect of second messenger modulators on noradrenaline release and synthesis

CAM kinase II The calmodulin-dependent protein (CAM) kinase II inhibitor, KN-62 (10 μM), inhibited the S-I fractional outflow of radioactivity from rat atria incubated in [^{3}H]-noradrenaline (Figure 4) without affecting the spontaneous fractional outflow of radioactivity (not shown). KN-62 (10 μM) did not alter S-I [^{3}H]-noradrenaline synthesis (Figure 4) or basal [^{3}H]-noradrenaline synthesis from [^{3}H]-tyrosine in rat atria (Table 1). Thus for KN-62, changes in S-I noradrenaline release are not matched with commensurate changes in S-I noradrenaline synthesis (Figure 4).

A separate series of experiments were designed to investigate whether KN-62 alters the release of [^{3}H]-noradrenaline induced by the calcium ionophore, A23187. In this case S_1 = electrical stimulation (3 Hz for 5 min) and S_2 = A23187 (20 μM) for 5 min. The outflow of radioactivity induced by A23187 in control experiments ($S_2/S_1 = 57.1\% \pm 8.3$, $n = 5$) was significantly greater ($P < 0.05$, Student's t test) than when KN-62 (10 μM) was present ($S_2/S_1 = 16.1\% \pm 7.2$, $n = 5$).

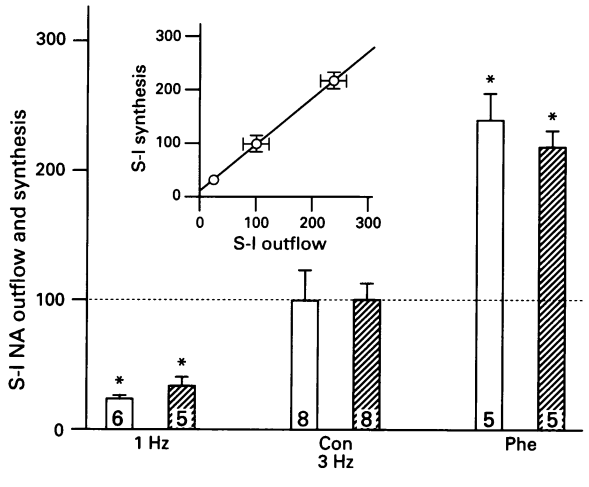

Figure 3 Comparison of stimulation-induced (S-I) endogenous noradrenaline (NA) outflow (open columns) and [^{3}H]-noradrenaline synthesis (hatched columns) after electrical field stimulation of rat isolated atria at 3 Hz for 5 min with treatments which alter Ca^{2+} entry into nerve terminals. The drugs and manipulations used at 3 Hz stimulation were: control (Con) and phentolamine (1 μM, Phe). In addition some atria were stimulated at 1 Hz (for 5 min). The noradrenaline outflow from the atria in control (3 Hz) experiments was measured by h.p.l.c. and was found to be 11.1 ± 2.5 pmol/atria. In separate experiments S-I [^{3}H]-noradrenaline synthesis was measured in atria incubated with [^{3}H]-tyrosine and was found to be 4.26 ± 0.57 pmol/atria ($n = 8$) based on the specific activity of the [^{3}H]-tyrosine in the bathing solution. All results were normalized such that 3 Hz control = 100. Means and s.e. mean are shown, and the number of observations (n) is at the base of each column. *Significant difference from the respective 3 Hz control experiments, $P < 0.05$, Student's t test with Bonferroni correction. Inset: there was a strong linear relationship between the amount of endogenous noradrenaline released on stimulation and the amount of [^{3}H]-noradrenaline synthesized in response to the stimulation: S-I synthesis = 0.9 × S-I release + 14.1; $r^2 = 0.99$, $P < 0.05$. See Table 1 for basal synthesis values.

Protein kinase A The combination of BrcyclicAMP (90 μM) and IBMX (100 μM) enhanced basal [^{3}H]-noradrenaline synthesis from [^{3}H]-tyrosine in rat atria (Table 1). These experiments were also repeated in rat right atrium so that the cyclic AMP antagonist RClcAMPS could be used. Similar to intact atria, BrcyclicAMP (90 and 270 μM) and the combination of BrcyclicAMP (90 μM) and IBMX (100 μM) enhanced basal [^{3}H]-noradrenaline synthesis from [^{3}H]-tyrosine in rat right atrium (Figure 5). In rat right atrium RClcAMPS (300 μM) significantly attenuated the enhancement of basal synthesis by BrcyclicAMP (270 μM) and the combination of BrcyclicAMP (90 μM) and RClcAMPS (100 μM) ($P < 0.05$, two-way ANOVA, Figure 5). RClcAMPS by itself, had no significant effect on basal [^{3}H]-noradrenaline synthesis (Figure 5).

The combination of BrcyclicAMP (90 μM) and IBMX (100 μM) slightly but significantly enhanced S-I outflow of radioactivity from rat atria incubated in [^{3}H]-noradrenaline and was without effect on S-I [^{3}H]-noradrenaline synthesis from [^{3}H]-tyrosine (Figure 4). RClcAMPS (300 μM) did not affect the S-I outflow of radioactivity from rat right atrium incubated with [^{3}H]-noradrenaline (Figure 4). In addition, RClcAMPS also had no effect on S-I [^{3}H]-noradrenaline synthesis suggesting no role for cyclicAMP (Figure 4).

Protein kinase C The protein kinase C inhibitor, polymyxin B (21 μM), and the non-selective protein kinase inhibitor, staurosporine (0.1 μM), inhibited the S-I fractional outflow of radioactivity from rat atria incubated in [^{3}H]-noradrenaline (Figure 4) without affecting the spontaneous fractional outflow of radioactivity (not shown). Both polymyxin B (21 μM) and staurosporine (0.1 μM) were without effect on basal (Table 1) and S-I (Figure 4) [^{3}H]-noradrenaline synthesis from [^{3}H]-tyrosine in rat atria. The protein kinase C activator, phorbol 12, 13-dibutyrate (0.1 μM), enhanced the S-I fractional outflow of radioactivity from rat atria incubated in [^{3}H]-noradrenaline (Figure 4) without affecting the spontaneous fractional outflow of radioactivity (not shown). Phorbol 12, 13-dibutyrate (0.1 μM) was also without effect on basal (Table 1) or S-I (Figure 4) [^{3}H]-noradrenaline synthesis from [^{3}H]-tyrosine in rat atria. For all of these protein kinase C drugs no relationship was observed between S-I noradrenaline release and synthesis since the changes in S-I transmitter release were not matched with commensurate changes in S-I synthesis (Figure 4).

Discussion

In the present study we examined the processes involved in the elevation of noradrenaline synthesis following a period of sympathetic nerve stimulation in rat atria. We used electrical stimulation at low frequencies for a short duration (1 and 3 Hz for 5 min) which is similar to the rate observed in single fibre nerve recordings *in vivo* (Sato *et al.*, 1986; Sato & Schaible, 1987). Electrical field stimulation (3 Hz for 5 min) of rat isolated atria incubated with [^{3}H]-noradrenaline resulted in the release of radioactivity which was used as an index of noradrenaline release. The biosynthesis of [^{3}H]-noradrenaline from [^{3}H]-tyrosine was also measured. The tissue displayed a basal biosynthetic activity for noradrenaline and after electrical stimulation the synthesis of noradrenaline was enhanced for up to 85 min with the bulk of the increase occurring in the first 45 min (Figure 1). Previous studies have also indicated sustained increases in synthesis after the cessation of nerve stimulation (e.g. Weiner & Rabadjija, 1968) and this most likely involves enhanced tyrosine hydroxylase activity since it is only

observed if radiolabelled tyrosine is employed to monitor synthesis and not when radiolabelled DOPA, the product of tyrosine hydroxylase, is used (Weiner & Rabadjija, 1968).

Both S-I noradrenaline release and S-I noradrenaline synthesis required extracellular Ca^{2+} and both were abolished (Figure 2) by the neuronal Na^+-channel blocker tetrodotoxin, suggesting that the S-I changes involved the conduction of action-potentials in sympathetic axons and Ca^{2+} entry at the nerve terminal. Similar findings of Ca^{2+}-dependency have been previously demonstrated (Boadle-Biber *et al.*, 1970) and N-type Ca^{2+}-channels have been implicated (Rittenhouse & Zigmond, 1991).

The role of Ca^{2+} was studied further by varying the amount of S-I noradrenaline release in a graded manner (Figure 2) by a variety of manipulations which involve alterations in Ca^{2+} entry into the nerve terminal either directly or indirectly: lowering the frequency of nerve stimulation to 1 Hz, by blocking repolarising K^+-channels with tetraethylammonium thus prolonging membrane depolarisation to allow more Ca^{2+} entry (Armstrong & Binstock, 1965) and by using adenosine which acts on presynaptic adenosine receptors (Fredholm *et al.*, 1990) and the α-adrenoceptor antagonist phentolamine, which prevents feedback inhibition of release by noradrenaline through presynaptic α-adrenoceptors (Starke *et al.*, 1989). These presynaptic receptors affect action-potential evoked noradrenaline release by inhibiting Ca^{2+} entry either through direct (G-protein) coupling to Ca^{2+}-channels (see De Langen & Mulder, 1980; Lipscombe *et al.*, 1989; Starke *et al.*, 1989) or indirectly by shortening Ca^{2+}-channel opening through activation of repolarising K^+ channels (see Starke *et al.*, 1989). In the presence of these drugs and manipulations there was a strong linear relationship between S-I noradrenaline release and S-I noradrenaline synthesis (Figure 2). We speculate from the close relationship between S-I noradrenaline synthesis and S-I noradrenaline release that there must be common elements in their signalling pathway. One possible explanation is that the noradrenaline which is released somehow stimulates transmitter synthesis, which would suggest that both release and synthesis share a common pathway of activation when Ca^{2+} enters the neurone. Alternatively, Ca^{2+} entry may set in train a series of events to alter synthesis which is independent of the release process and the close relationship is only evident because the common trigger is Ca^{2+} entry.

In order to test this latter hypothesis we sought to alter noradrenaline release by altering the release process subsequent to Ca^{2+} entry. A prime target is CAM kinase II an intraneuronal enzyme which is activated by Ca^{2+}/calmodulin

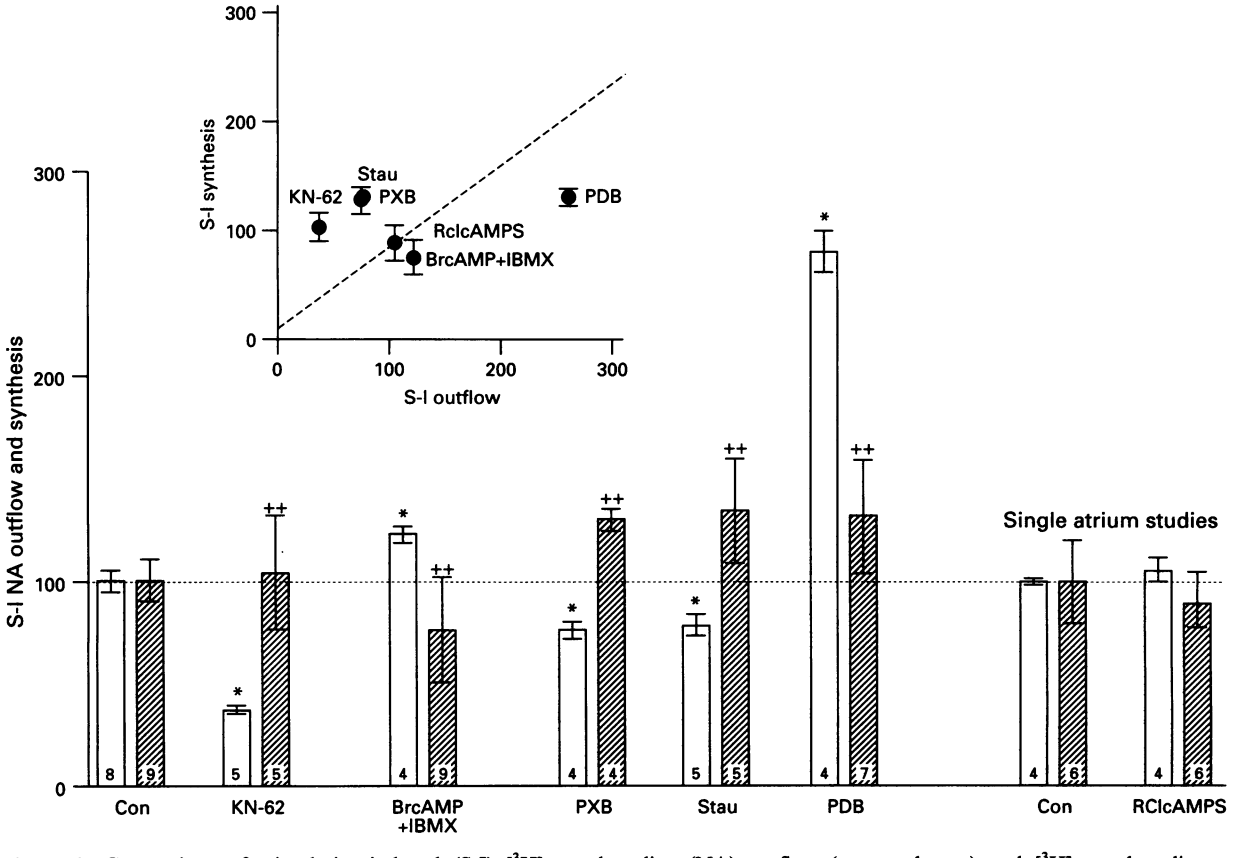

Figure 4 Comparison of stimulation-induced (S-I) [^{3}H]-noradrenaline (NA) outflow (open columns) and [^{3}H]-noradrenaline synthesis (hatched columns) after electrical field stimulation of rat isolated atria (3 Hz for 5 min) and with drugs which alter second messenger systems. The drugs and manipulations used at 3 Hz stimulation were: control (Con), KN-62 (10 μM), the combination of 8-bromoadenosine 3', 5'-cyclic monophosphate (BrcAMP) and 3-isobutyl-1-methyl-xanthine (90 μM, BrcAMP + 100 μM, IBMX), polymyxin B (21 μM, PXB), staurosporine (0.1 μM, Stau) and phorbol 12, 13-dibutyrate (0.1 μM, PDB). For Rp-8-chloroadenosine 3', 5'-cyclic monophosphorothioate (RClcAMPS, 300 μM), the experiments were carried out using one atrium in each case to conserve drug, and concurrently controls were also carried out in a single atrium. The S-I noradrenaline outflow was measured in tissue incubated with [^{3}H]-noradrenaline and for 3 Hz control experiments; for atria studies $FR_2/FR_1 = 0.93 \pm 0.04$ ($n = 8$) and for single atrium studies $FR_2/FR_1 = 0.91 \pm 0.05$ ($n = 4$). In separate experiments S-I [^{3}H]-noradrenaline synthesis was measured in tissue incubated with [^{3}H]-tyrosine and was found to be 3.40 ± 0.80 pmol/atria ($n = 9$) for atria studies and 3.62 ± 0.76 pmol/atria ($n = 6$) for single atrium studies, based on the specific activity of the [^{3}H]-tyrosine in the bathing solution. All results were normalized such that 3 Hz control = 100. Means and s.e. mean are shown, and the number of observations (n) is at the base of each column. *Significant difference from the respective 3 Hz control experiments, $P < 0.05$, Student's t test with Bonferroni correction. ++ Significant difference between release and synthesis pairs, $P < 0.05$, Student's t test. Inset: The regression line refers to that of Figure 2 for Ca^{2+}-dependent alterations in transmitter release and synthesis and the second messenger drugs are represented individually. See Table 1 for basal synthesis values.

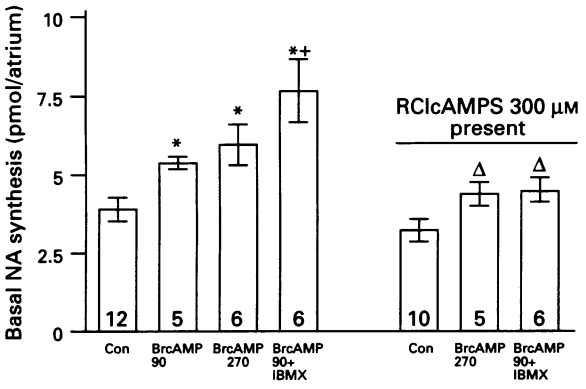

Figure 5 Modulation of basal [^{3}H]-noradrenaline (NA) synthesis from [^{3}H]-tyrosine in rat right atrium by activators and an inhibitor of cyclic AMP-dependent protein kinase A. These experiments were carried out in one atrium in each case to conserve the drug, Rp-8-chloroadenosine 3', 5'-cyclic monophosphorothioate (RClcAMPS), and concurrently controls were also carried out in a single atrium. Means and s.e. mean are shown, and the number of observations (n) is at the base of each column. *Significant difference from control, $P<0.05$, Student's t test with Bonferroni correction. $^+$Significant difference from BrcAMP (90 μM), $P<0.05$., Student's t test. $^\Delta$ In the presence of RClcAMPS (300 μM) the effects of 8-bromoadenosine 3', 5'-cyclic monophosphate (BrcAMP, 270 μM) and the combination of 8-bromoadenosine 3', 5'-cyclic monophosphate and 3-isobutyl-1-methyl-xanthine (90 μM, BrcAMP + 100 μM, IBMX) were significantly attenuated ($P<0.05$, two-way ANOVA).

and phosphorylates key vesicle associated proteins to induce transmitter release (see DeLorenzo, 1981; Huttner *et al.*, 1981; Bahler *et al.*, 1991; Rubenstein *et al.*, 1993). We used KN-62 to inhibit CAM kinase II (Tokumitsu *et al.*, 1990). As a test that KN-62 acted independently of voltage-dependent Ca^{2+} entry, we examined the effect of KN-62 on release induced by the ionophore A23187, which inserts into membranes and due to its physicochemical properties allows the passage of Ca^{2+} to induce release (Pressman, 1976). The release of noradrenaline induced by A23187 is not modulated by pathways involving Ca^{2+} entry through ion channels (e.g. presynaptic α-adrenoceptors, De Langen & Mulder, 1980). However, in line with the proposed role for CAM kinase II in vesicle dynamics, KN-62 substantially inhibited the A23187-induced noradrenaline release suggesting that it affected release by altering the ability of intraneuronal Ca^{2+} to induce release. KN-62 also markedly decreased electrical S-I noradrenaline release in rat atria (Figure 4) as has also been observed in brain noradrenergic neurones (Dunlop *et al.*, 1993). However, KN-62 had no effect on S-I noradrenaline synthesis (Figure 4). This dissociation between S-I noradrenaline release and S-I synthesis with KN-62 supports our hypothesis that Ca^{2+} entry into the nerve terminal or events closely coupled to it determines the level of synthesis rather than down-stream release events or the released transmitter itself.

The close relationship between S-I noradrenaline synthesis and S-I noradrenaline release for Ca^{2+}-dependent processes discussed above is unlikely to be explained by activation of presynaptic receptors, or the direct actions of the drugs on the synthetic machinery, since basal synthesis was not affected by the various manipulations (Ca^{2+} free medium, tetrodotoxin, tetraethylammonium, adenosine, phentolamine). Importantly, basal synthesis was not altered by the α-adrenoceptor agonist clonidine which agrees with Birch & Fillenz (1985). Thus automodulation cannot explain the present findings. This contrasts with the dopaminergic system in the striatum where activation of DA_1-receptors *per se* increases dopamine synthesis (e.g. Onali *et al.*, 1988).

Second messenger activated pathways have been suggested previously to elevate noradrenaline biosynthesis (see Introduction). Indeed, in the present study BrcyclicAMP enhanced basal noradrenaline biosynthesis (Figure 5) in the presence and absence of the phosphodiesterase inhibitor IBMX. Similar findings have been made previously in rat atria and in other sympathetically innervated tissues in accord with cyclic AMP stimulation of noradrenaline biosynthesis (Schwarzschild & Zigmond, 1991). This action of cyclic AMP is probably due to phosphorylation of tyrosine hydroxylase (Campbell *et al.*, 1986; Haycock, 1990). The cyclic AMP antagonist RClcAMPS (Schaap *et al.*, 1993), significantly attenuated the actions of BrcyclicAMP on basal noradrenaline synthesis (Figure 5) but had no effect itself on basal noradrenaline synthesis (Figure 5), indicating no basal effect of endogenous cyclic AMP. Importantly, RClcAMPS did not inhibit the S-I increase in noradrenaline synthesis (Figure 4) which suggests no role for endogenous cyclic AMP in the signalling pathway for S-I noradrenaline synthesis in rat atria. In addition staurosporine, which can also inhibit protein kinase A (Tamaoki *et al.*, 1986), was also without effect on S-I noradrenaline synthesis. Staurosporine is a non-selective kinase inhibitor and also blocks CAM kinase II (Yanagihara *et al.*, 1991) and its lack of effect on basal and S-I noradrenaline synthesis, like the other CAM kinase II inhibitor KN-62, also argues against the hypothesis that CAM kinase II phosphorylation of tyrosine hydroxylase is involved in the activation of noradrenaline synthesis as previously proposed (Knor *et al.*, 1986; Ishii *et al.*, 1991).

Protein kinase C has been implicated in the phosphorylation and activation of tyrosine hydroxylase (see Introduction). However, in disagreement with this hypothesis, the large increases in S-I noradrenaline release with the protein kinase C activator phorbol 12, 13-dibutyrate occurred without any change in S-I noradrenaline synthesis (Figure 4). Consistent with this, the protein kinase C inhibitors, polymyxin B and staurosporine (Schachtele *et al.*, 1988), decreased S-I noradrenaline release without affecting S-I synthesis (Figure 4). These results not only rule out protein kinase C phosphorylation of tyrosine hydroxylase as being physiologically relevant but also give clues as to the actions of protein kinase C on transmitter release. We propose that synthesis is activated by Ca^{2+} entering the nerve terminal, therefore the ability of protein kinase C activators and inhibitors to alter transmitter release without altering synthesis indicate that protein kinase C alters noradrenaline release subsequent to Ca^{2+} entry. Indeed, the release of noradrenaline from sympathetic nerves induced by the calcium ionophore A23187 was enhanced by phorbol esters (Ishac & De Luca, 1987; Hashimoto *et al.*, 1988) and protein kinase C substrates have been implicated in vesicle dynamics (Dekker *et al.*, 1989).

It has been suggested that S-I increases in noradrenaline synthesis are the result of reduced feedback inhibition of noradrenaline on tyrosine hydroxylase due to decreased intraneuronal noradrenaline following noradrenaline release (see Weiner *et al.*, 1972). However, for four pharmacological manipulations (KN-62, phorbol 12, 13 dibutyrate, polymyxin B and staurosporine) changes (enhancement and reductions) in noradrenaline release were not accompanied by commensurate changes in synthesis. This observation rules out the possibility that S-I synthesis is determined directly by the amount of noradrenaline released or procedures set in train by the noradrenaline that is released.

Noradrenaline synthesis or parameters derived from synthesis have been used as an index of noradrenaline release *in vivo* (Schmid *et al.*, 1986). This may be valid for long term changes which depend on the induction of tyrosine hydroxylase (see Zigmond, 1989). However, data from the present study suggest that acute changes in noradrenaline release and synthesis are only related where the changes in release are brought about by an alteration in Ca^{2+} entry into the nerve terminal. Systems which act intracellularly to alter the sensitivity of the release process to Ca^{2+}, such as the signal transduction manipulations detailed above, produce changes in action-potential evoked noradrenaline release without commensurate changes in noradrenaline synthesis.

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