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RELATION BETWEEN CATECHOLAMINE-INDUCED CYCLIC AMP CHANGES AND HYPERPOLARIZATION IN ISOLATED RAT SYMPATHETIC GANGLIA

BY D. A. BROWN, M. P. CAULFIELD AND P. J. KIRBY*

*From the Department of Pharmacology, The School of Pharmacy, University of London, 29/39 Brunswick Square, London WC1N 1AX and the * Department of Clinical Pharmacology, Cardiothoracic Institute, Fulham Road, London SW3 6HP*

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SUMMARY

1. The effect of catecholamines on cyclic adenosine 3′5′-monophosphate (cyclic AMP) production in isolated rat superior cervical ganglia has been measured under experimental conditions in which they also produce ganglion hyperpolarization.

2. (±)Isoprenaline (1 μM) increased cyclic AMP levels by 8–100 times after 15 min incubation at 25 °C. Half-maximal stimulation occurred at about 0·03 μM. This was due to stimulation of β-receptors, since it was prevented by 1 μM-propranolol but not by 1 μM-phentolamine.

3. The α-agonists phenylephrine (100 μM), dopamine (100 μM) and clonidine (1 μM) did not produce a detectable increase in ganglionic cyclic AMP. Dopamine (100 μM) was also ineffective at 37 °C in the presence of 10 mM-theophylline.

4. Exogenous cyclic AMP (0·01–1 mM) hyperpolarized the ganglion. This effect was replicated by other adenosine compounds, most effectively by adenosine and by adenosine 5′-monophosphate, and was antagonized by theophylline. Dibutyryl cyclic AMP was weaker than cyclic AMP.

5. Neither theophylline nor the non-xanthine phosphodiesterase inhibitor, Ro 20-1724, enhanced the hyperpolarizing actions of noradrenaline or dopamine.

6. Since catecholamine-induced hyperpolarization of the isolated rat ganglion is induced via α-receptors, whereas cyclic AMP-production is induced via β-receptors, it is concluded that cyclic AMP is unlikely to mediate the hyperpolarization. The effect of exogenous cyclic AMP may be due to an action on external adenosine-receptors.

INTRODUCTION

It has been suggested that the hyperpolarization of sympathetic ganglion cells produced by catecholamines may result from elevation of intraneuronal cyclic adenosine-3′5′-monophosphate (cyclic AMP). Thus, Kebabian & Greengard (1971) described a 'dopamine-sensitive' adenyl cyclase in bovine sympathetic ganglia; and McAfee & Greengard (1972) reported that the hyperpolarization of isolated rabbit superior cervical ganglia produced by dopamine (previously described by Libet & Tosaka (1970)) was potentiated by the phosphodiesterase-inhibitor, theophylline, and

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replicated by exogenous cyclic AMP or its butyryl derivatives. These observations led Greengard (1976) to propose that ganglionic hyperpolarization by catecholamines was mediated by activation of a dopamine-receptor linked adenyl cyclase.

However, correlation between biochemical observations in one species and electrophysiological observations in another is unsatisfactory, particularly since considerable variation has been found in the amounts of cyclic AMP formed by different catecholamines in the ganglia of different mammalian species (Black, Chiba, Wamsley & Williams, 1976). Indeed, dopamine appears to produce hardly detectable increases ($15 \pm 4\%$) in cyclic AMP in rabbit ganglia (McAfee, 1976). Hence, a careful comparison of cyclic AMP changes and ganglionic hyperpolarization in the same tissue seems mandatory.

Recently, we (Brown & Caulfield, 1979) have obtained a fairly precise characterization of the adrenoceptors responsible for hyperpolarization of rat superior cervical ganglia. They are not dopamine-receptors, but instead resemble the 'α_2' subclass of α-receptors (see Berthelsen & Pettinger, 1977). In contrast, previous reports suggest that rat ganglion adenyl cyclase is preferentially activated via β-receptors (Cramer, Johnston, Hanbauer, Silberstein & Kopin, 1973; Lindl & Cramer, 1975). Since β-receptor activation does not produce ganglion hyperpolarization (De Groat & Volle, 1966; Brown & Caulfield, 1979), the biochemical and electrical events in the rat ganglion may not be associated. If confirmed, this would clearly negate the general validity of cyclic AMP as a second messenger responsible for catecholamine-induced ganglionic hyperpolarization.

The present experiments were carried out to test whether there is an elevation in cyclic AMP in isolated rat superior cervical ganglia following α_2-receptor activation, using experimental conditions approximating as closely as possible those in which α_2-mediated hyperpolarizations were recorded. We have also tested cyclic AMP and phosphodiesterase-inhibitors on this preparation, to see whether they mimicked or potentiated respectively the hyperpolarization effects of catecholamines.

METHODS

Superior cervical sympathetic ganglia were removed from adult male Wistar rats (200–300 g weight) anaesthetized with urethane (1·5 g/kg I.P.). The outer connective tissue sheaths were removed and the desheathed ganglia were stored overnight at 4 °C in Krebs solution which had been bubbled with 95% O_2/5% CO_2 gas mixture (this overnight storage duplicates the conditions under which catecholamine-induced hyperpolarization was measured: Brown & Caulfield, 1979).

Cyclic AMP measurements

Ganglia were incubated individually in small baths at 25 °C containing 1·4 ml. bubbled Krebs solution for 60 min. Any antagonists used were present during this pre-incubation period. Drugs (or Krebs solution, in the case of controls) were injected into the bath as 100 μl. volumes (using a microsyringe) and after the desired incubation time the bath containing the ganglion (together with incubation medium) was put in a boiling water bath for 5 min. The ganglion was then homogenized in 0·5 ml. ice-cold buffer, the homogenate recombined with the incubation fluid and the resulting mixture centrifuged at 10,000 rev/min. for 10 min (at 4 °C). The supernatant was taken and kept on ice until required. By taking both incubation fluid and ganglion for assay of cyclic AMP, account would be taken of any cyclic AMP released into the medium (see Lindl & Cramer, 1974).

Columns (0·8 × 2·5 cm) were prepared in small separating funnels using Dowex AG1–× 8 resin (formate form: mesh 200–400; Biorad) which had previously been equilibrated with de-ionized, distilled water. After draining the column until the meniscus was level with the top of the resin, the supernatant (2 ml.) was applied to the column. The columns were washed with 10 ml. distilled water, then with 12 ml. 2 N formic acid to elute cyclic AMP (cyclic GMP remaining bound). This eluate was collected and freeze-dried; the deposit was redissolved in 200 μl. phosphate–citrate buffer (pH 5·8) on ice. 50 μl. of this was taken for assay. Recovery to this stage was estimated by adding a small amount (0·02 μc = 0·63 p-mole) of [^{3}H]cyclic AMP (Radiochemical Centre) to the original supernatant. Recoveries were found to be between 66 and 75% and the estimates of cyclic AMP were corrected accordingly.

Cyclic AMP was assayed using a modification (Connolly & Greenacre, 1977) of the protein binding procedure of Gilman (1970). The binding protein was a protein kinase obtained from rabbit skeletal muscle separated using a protamine-sepharose affinity chromatography column. The assay incubation mixture comprised (*a*) 50 μl. phosphate–citrate buffer (pH 5·8) containing either the sample aliquot, or known amounts of cyclic AMP (1–30 p-mole) for calibration purposes, (*b*) 0·5 p-mole [^{3}H]cyclic AMP and (*c*) 50 μl. binding protein, previously diluted 1:1 with bovine serum albumin, 1 mg/ml. The assay mixture was thoroughly stirred then left on ice at 4 °C for 2 hr, after which the solution was put onto Millipore filters (HAWP 02400, 0·45 μM pore size) which were rinsed with ice-cold 20 mM-phosphate buffer (pH 6·6) under suction. The filters were dissolved in 1 ml. 2-ethoxyethanol (in counting vials), then counted for radioactivity after addition of scintillant. In this method any 'cold' cyclic AMP in the sample competes for binding to the protein with the radioactive cAMP; the protein (+bound cyclic AMP) is trapped on the filters, and hence the greater the amount of 'cold' cyclic AMP, the less radioactivity (as [^{3}H]cyclic AMP) is present on the filters. The relationship between the amount of 'cold' cyclic AMP present, and the binding of radioactive cyclic AMP was found by constructing a plot of log (disintegrations per minute (dpm) on filters) *vs.* log (cyclic AMP). This relationship was close to linearity at 1–30 p-mole cyclic AMP and least-squares regression analysis was used to find the best fit for the calibration plot; the amount of cyclic AMP in the sample was then found by substitution in the regression equation.

Ganglion hyperpolarization

Hyperpolarization was measured with an extracellular air-gap method using superfused ganglia (Brown & Marsh, 1975), with the modifications described by Brown & Caulfield (1979) to ensure constant temperature (25 °C) and d.c. stability. The superfusion rate was 1 ml./min; drugs were applied to the ganglion through separate flow-lines without premixing and arrival timed with an air-bubble. Responses were recorded potentiometrically as surface-positive deflexions at the ganglion with respect to the reference electrode on the internal carotid post-ganglionic nerve.

Krebs solution

The solution had the following composition (mM): NaCl, 118; KCl, 4·8; $CaCl_2$, 2·5; $NaHCO_3$, 25; KH_2PO_4, 1·18; $MgSO_4$, 1·19; D-glucose, 11 (pH 7·4).

Compounds

The following compounds were used (with sources): cyclic 3′5′-adenosine monophosphate (cyclic AMP, Na salt), adenosine 5′-triphosphate (ATP, Na salt), adenosine 5′-diphosphate (ADP, Na salt), adenosine 5′-monophosphate (AMP, Na salt), (±)adenosine, (−)noradrenaline bitartrate, (±)isoprenaline hydrochloride, dopamine hydrochloride, (−)phenylephrine hydrochloride, (±)propranolol hydrochloride (all from Sigma); phentolamine methanesulphonate (Rogitine, from CIBA); and theophylline hydrate (B.D.H.).

Cyclic N^6-2′-*O*-dibutyryl adenosine 3′5′-cyclic monophosphate (dibutyryl cyclic AMP) and the phosphodiesterase inhibitor Ro 20-1724 (4-(3,4-dibutoxybenzyl)-2-imidazolidinone) were a gift from Dr L. L. Iversen.

RESULTS

Cyclic AMP measurements

β-Agonists. (±)Isoprenaline (1 μM) increased the amount of cyclic AMP in the ganglion and incubation medium combined, from a resting level of 12·7 ± 3·7 p-mole/ganglion (mean ± S.E.; $n = 4$) to 104·5 ± 16·4 p-mole/ganglion after 15 min incubation at 25 °C (Fig. 1). Half-maximal stimulation of cyclic AMP production after 15 min incubation occurred at about 0·03 μM (Fig. 2).

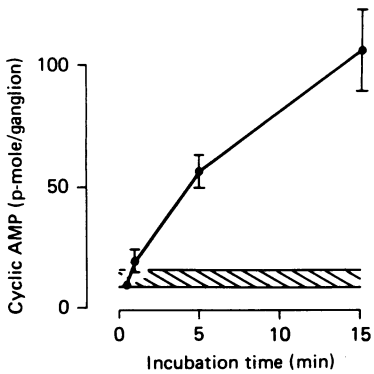

Fig. 1. Effect of 1 μM-(±)isoprenaline on cyclic AMP levels in isolated rat superior cervical ganglia. The ganglia were incubated with isoprenaline for increasing periods of time at 25 °C and the amounts of cyclic AMP in the ganglia and in the incubation medium measured (see Methods). Points and bars show means and standard errors of means respectively (four ganglia for each point). The shaded area shows the mean and standard error for four control ganglia incubated without added isoprenaline.

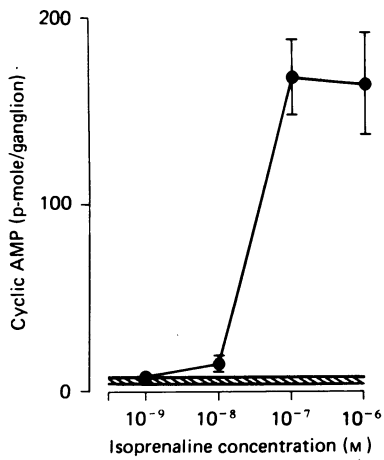

Fig. 2. Effect of varying concentrations of (±)isoprenaline on ganglionic cyclic AMP levels measured after 15 min incubation at 25 °C (see Fig. 1 for details).

The β-receptor blocking agent, propranolol (1 μM), reduced the sensitivity to isoprenaline by a factor of at least 100 (Fig. 3). In contrast, the α-receptor blocking agent phentolamine (1 μM) did not clearly reduce the amount of cyclic AMP produced by isoprenaline.

α-Agonists. In concentrations which produce near-maximal rat ganglion hyperpolarization (q.v., Brown & Caulfield, 1979), incubation of ganglia for 15 min with the α-agonists phenylephrine (100 μM), dopamine (100 μM) and clonidine (1 μM) did not significantly increase ganglionic cyclic AMP levels (Table 1). In the same experiment, 1 μM-isoprenaline increased cyclic AMP by about 100 times.

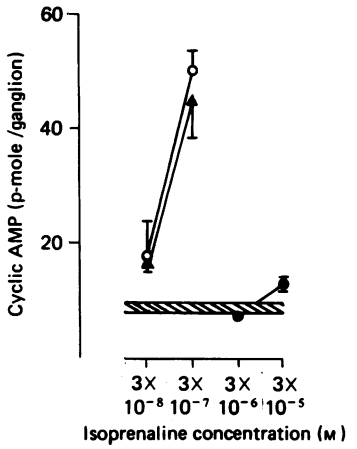

Fig. 3. Effect of 15 min incubation with (±)isoprenaline alone (○), or in the presence of 1 μM-phentolamine (▲) or 1 μM-(±)propranolol (●). Shaded area shows mean and S.E. of mean of four control ganglia, not incubated with isoprenaline; points and bars are means and S.E. respectively of four ganglia. The antagonists were added to the incubation medium 60 min before adding isoprenaline.

TABLE 1. Effect of sympathomimetic amines on cyclic AMP levels in isolated rat superior cervical ganglia. Two series of experiments were performed, at 25 and 37 °C, respectively. Agonists were applied for 15 min; theophylline where used in the second series, was applied 60 min before the agonist. Each value listed is the mean (± S.E.) of four ganglia, except for those marked *, which are single determinations

Temp. (°C)	Agonist	Conc.	Cyclic AMP (p-mole/ganglion)
25	None	—	1·7 ± 0·5
	(−)Phenylephrine	100 μM	3·3 ± 0·7
	Dopamine	100 μM	1·5 ± 0·4
	Clonidine	1 μM	1·24 ± 0·43
	(±)Isoprenaline	1 μM	174*
37	None	—	0·8 ± 0·14
	Dopamine	100 μM	0·87 ± 0·19
	Theophylline	10 μM	6·8 ± 1·3
	Theophylline + dopamine	10 mM + 100 μM	8·8 ± 1·8
	(±)Isoprenaline	1 μM	22·4*
	Theophylline + (±)isoprenaline	10 mM + 1 μM	267*

In a second series of experiments conducted at 37 °C dopamine (100 μM, 15 min incubation) remained ineffective, even when tested on ganglia pretreated with the phosphodiesterase-inhibitor, theophylline (10 mM), (Table 1). Theophylline alone raised the amount of cAMP measured after incubation in 0·1 μM-isoprenaline

by about 10 times. Hence, the previous ineffectiveness of dopamine could not be ascribed either to the low incubation temperature or to excessive phosphodiesterase activity.

Potential changes produced by exogenous cyclic AMP

Exogenous cyclic AMP (0·01–1 mM) hyperpolarized the isolated rat superior cervical ganglion (Fig. 4). The hyperpolarization produced by 1 mM-cyclic AMP was

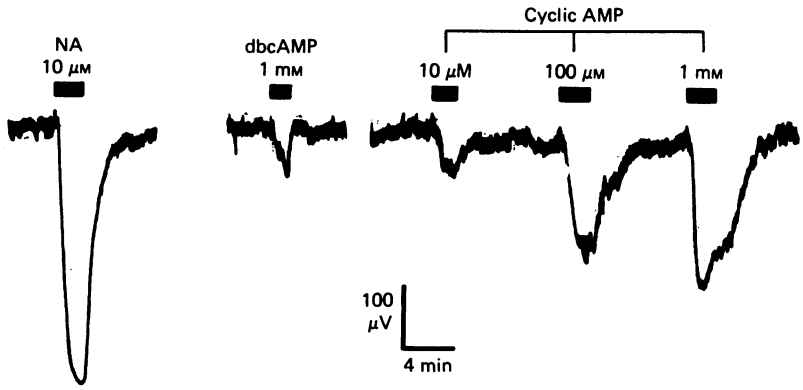

Fig. 4. Hyperpolarizations of an isolated rat superior cervical ganglion produced by 10 μM-(−)noradrenaline (NA), 1 mM-dibutyryl cyclic AMP (dbcAMP), and by 10 μM, 100 μM and 1 mM-cyclic AMP. Drugs were applied for the periods indicated by the horizontal bars, by superfusion (see Methods), at intervals not less than 10 min.

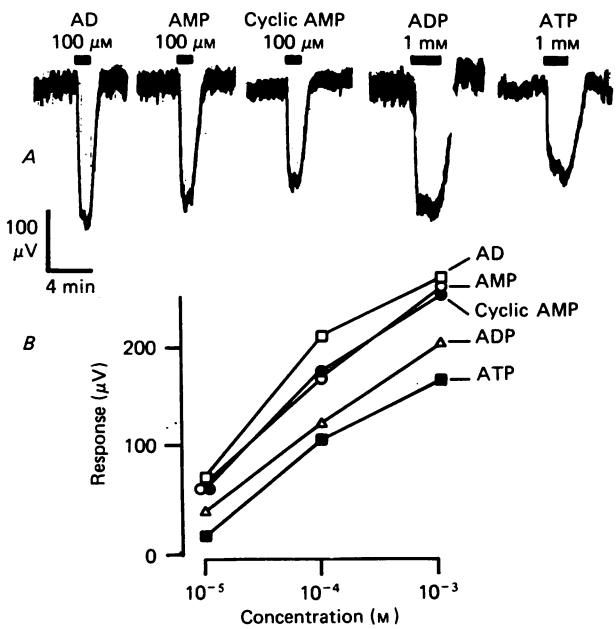

Fig. 5. The upper trace (*A*) shows hyperpolarizing effects of adenosine (AD, 100 μM), adenosine 5′-monophosphate (AMP, 100 μM), cyclic AMP (100 μM), adenosine 5′-diphosphate (ADP, 1 mM) and adenosine 5′-triphosphate (ATP, 1 mM), recorded in the same ganglion at intervals not less than 10 min. The lower part of the Figure (*B*) shows dose–response curves to adenosine compounds recorded in a single ganglion.

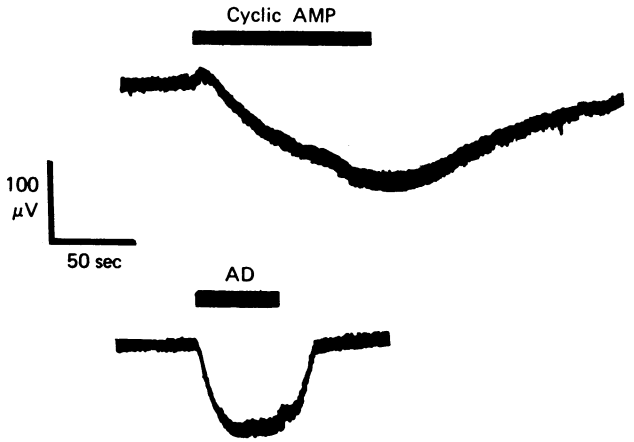

Fig. 6. Comparative time-courses for onset and offset of ganglion hyperpolarization produced by 100 μM-cyclic AMP (upper record) and 30 μM-adenosine (AD, lower record), recorded in the same preparation.

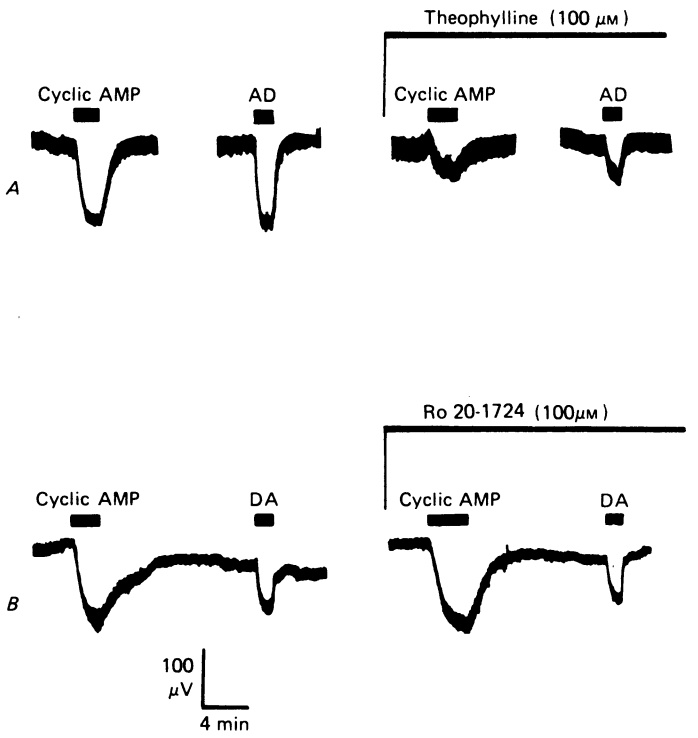

Fig. 7. Effects of (*A*) 100 μM-theophylline and (*B*) 100 μM Ro 20-1724 on hyperpolarizing responses to 100 μM-cyclic AMP, 50 μM-adenosine (AD) and 100 μM-dopamine (DA) (results from two preparations).

smaller than that evoked by 10 μM-noradrenaline, and was also slower in onset and offset (see also Fig. 6). Dibutyryl cyclic AMP was less effective than cyclic AMP itself, notwithstanding the former's greater resistance to phosphodiesterase (Cheovic, Posternak & Charollais, 1972).

The action of cyclic AMP was imitated by some non-cyclic adenosine compounds, most noticeably by adenosine and AMP (Fig. 5). The relative activity of the compounds tested diminished in the order adenosine > cyclic AMP $\approx$ AMP > ADP > ATP > dibutyryl cyclic AMP.

The rate of onset of hyperpolarization following addition of cyclic AMP was noticeably slower than that following other adenosine compounds (Fig. 6).

Phosphodiesterase inhibitors. Theophylline (10–100 μM) *depressed* the hyperpolarizing responses to cyclic AMP and to adenosine (Fig. 7). At higher concentrations theophylline depolarized the ganglion and reduced the response to nicotinic agonists. The non-xanthine inhibitor Ro 20-1724 (Sheppard, Wiggan & Tsien, 1972) did not potentiate responses to cyclic AMP at 100 μM (Ro 20-1724 increases resting and evoked cyclic AMP levels in rabbit ganglia at this concentration: Kalix, McAfee, Schorderet & Greengard, 1974).

Neither theophylline (100 μM) nor Ro 20-1724 (100 μM) increased the ganglionic hyperpolarization produced by noradrenaline or dopamine (Fig. 7*A*).

DISCUSSION

The principal significance of the present experiments lies in the attempt to relate catecholamine-induced ganglionic hyperpolarization and changes in cyclic AMP levels in the same tissue (the rat superior cervical ganglion) under similar experimental conditions. The results clearly show that no such relationship exists. Whereas hyperpolarization results from α_2-receptor stimulation (Brown & Caulfield, 1979), rat ganglion adenyl cyclase is coupled with β-receptors. In this we confirm the previous observations of Cramer *et al.* (1973) and Lindl & Cramer (1975). More to the point, no significant increase in ganglionic cyclic AMP could be detected after α_2-receptor stimulation sufficient to produce maximal ganglionic hyperpolarization, even in the presence of excess theophylline. It is, of course, possible that the hyperpolarization is triggered by a highly localized generation of cyclic AMP, to small and perhaps too labile to be registered as an increase in total cyclic AMP. The failure of phosphodiesterase inhibitors to potentiate the hyperpolarization action of catecholamines on the rat ganglion suggest this to be unlikely; in any case, such arguments would also negate the value of the previously demonstrated elevations in cyclic AMP in many other tissues as evidence for mediation by cyclic AMP.

In the isolated rat ganglion, β-receptor activation produces neither a potential change (Brown & Caulfield, 1979) nor a clear effect on the transmission of preganglionic nerve impulses (Caulfield, 1978). The large elevation in cyclic AMP produced by β-agonists therefore seems to have no immediate electrophysiological consequence. It may be that the β-coupled adenyl cyclase is localized to non-neural elements (Gilman & Nirenberg, 1971; Kebabian, Bloom, Steiner & Greengard, 1975), though the experiments of Otten, Mueller, Oesch & Thoenen (1974) suggest that this cannot be a very large fraction of the total isoprenaline-sensitive pool of adenyl cyclase in the rat. Alternatively, if neuronal in location, cyclic AMP might produce

long-term changes in excitability of the type observed by Kobayashi, Hashiguchi & Ushiyama (1978) to follow intraneuronal injections of cyclic AMP into rabbit ganglion cells.

The ganglion hyperpolarization produced by cyclic AMP is probably mediated through an action on external 'adenosine receptors', of the type described previously in nervous tissue (see, for examples, Sattin & Rall, 1970; Ginsborg & Hirst, 1972; Phillis, Kostopoulos & Limacher, 1974; Scholfield, 1978; Hayashi, Mori, Yamada & Kunitomo, 1978). Thus, the action of cyclic AMP was replicated by adenosine and AMP, and blocked by theophylline. It is unclear whether cyclic AMP acts directly on adenosine receptors, or indirectly through transformation to AMP or adenosine: although the slow response to cyclic AMP might suggest the latter, no substansti-ating evidence was obtained using phosphodiesterase inhibitors.

It seems very unlikely that any substantial part of the hyperpolarization produced by cyclic AMP resulted from intracellular effects following membrane penetration, since the hyperpolarization was not potentiated by phosphodiesterase inhibitors, and the dibutyryl derivative was *less* effective than cyclic AMP, rather than more effective. This accords with several recent reports that intracellularly injected cyclic AMP also fails to hyperpolarize sympathetic neurones (Gallagher & Shinnick-Gallagher, 1977; Kobayashi *et al.* 1978; Busis, Schulman, Smith & Weight, 1978*a*). The original report that *externally* applied cyclic AMP hyperpolarizes ganglion cells (McAfee & Greengard, 1972) was confirmed by Machova & Kristofova (1973), but not by more recent studies (Akasu & Kotesu, 1977; Dun & Karczmar, 1977; Dun, Kaibara & Karczmar, 1977; Busis *et al.* 1978*b*). Although in apparent accord with McAfee & Greengard's (1972) report, the present experiments suggest that, even where a hyperpolarization is detectable, it may have little connexion with cate-cholamine-induced hyperpolarization.

To sum up, cyclic AMP is not likely to mediate catecholamine-induced hyper-polarization in rat sympathetic ganglia. Since the original hypothesis stemmed from experiments on bovine and rabbit ganglia, it might be argued that this merely reflects a species difference. However, it should be noted that no detailed comparison of biochemical and electrophysiological responses to catecholamines has yet been completed in rabbit ganglia; that the limited information available suggests a poor correlation (McAfee, 1976); and other evidence contrary to a cyclic AMP-mediated hyperpolarization in rabbit ganglia has been described (Dun & Karczmar, 1977; Dun *et al.* 1977). At the the very least, the present results indicate the cyclic AMP hypothesis for ganglion hyperpolarization to be of limited applicability.

Finally, we have previously drawn attention to the analogy between the hyper-polarization of rat ganglion cells produced by catecholamines and their inhibitory effect on transmitter release from adrenergic nerve terminals (Brown & Caulfield, 1979). This analogy extends not only to the comparability of the adrenoceptors involved, but also to their sensitivity to Ca^{2+} and K^+ ions. If the two processes are indeed related, the present experiments might imply that changes in cyclic AMP content in nerve terminals are unlikely to be responsible for 'negative-feedback' inhibition of noradrenaline release (see Starke, 1977).

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