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Adenosine reduces agonist-induced production of inositol phosphates in rat aorta

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In rat aortic strips rendered permeable with digitonin, inositol trisphosphate induced an efflux of ^{45}Ca from the tissue. This release was not affected by adenosine. In tissues not treated with digitonin the contents of inositol trisphosphate (IP_3) and its metabolite inositol 1-phosphate (IP_1) were significantly enhanced by noradrenaline in the lithium-treated rat aorta. Adenosine was without effect on levels of IP_1 or IP_3 in tissues which had not been pretreated with noradrenaline, however, the noradrenaline-enhanced tissue content of IP_1 was reduced by adenosine in a dose-dependent manner. The reduction in IP_1 content by adenosine was enhanced by the uptake blocker dipyridamole (10 μM) and was blocked by the adenosine receptor antagonist 8-phenyltheophylline (10 μM). Adenosine may therefore lower production of inositol phosphates and thus reduce the stimulated release of calcium from intracellular stores. It is proposed that a reduction in phosphatidylinositol turnover may play a role in adenosine-mediated relaxation of blood vessels.

Adenosine has been recognized as possessing vasodilatory activity since Drury & Szent-Gyorgi (1929) demonstrated that this purine lowered arterial blood pressure to a degree which could not be entirely explained by a reduction in cardiac output. Adenosine actions on vascular muscle have been attributed to a reduction in cellular calcium uptake (Schnaar & Sparks 1972; Belardinelli et al 1979; van Breemen & Casteels 1974; Schrader et al 1975). Direct measurements of calcium fluxes have been performed by Guthrie & Naylor (1967) using mammalian atria to demonstrate a decrease in inward calcium flux with adenosine, and by Dutta et al (1980) who were unable to show any effect on calcium uptake in coronary artery. In view of this confusion we have considered the possibility that adenosine may modify calcium availability indirectly, by interfering with intracellular second messengers which affect calcium influx or intracellular release. An increased turnover of phosphoinositides has been described in non-muscle cells in response to stimulation by secretagogues (Hokin & Hokin 1953; Michell 1975) ultimately increasing the formation of inositol 1,4,5-trisphosphate (IP_3) which has subsequently been shown to release calcium ions from endoplasmic reticulum (Streb et al 1983; Berridge & Irvine 1984). The increased formation of IP_3 has been shown to occur in vascular smooth muscle in response to pharmacomechanical stimulation such as noradrenaline (NA) (Legan et al 1985; Campbell et al 1985).

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In the present study we have therefore sought to determine the effects of adenosine on inositol phosphate turnover as well as on the stimulation of calcium release from intracellular sites by inositol phosphates.

MATERIALS AND METHODS

Male Wistar rats (200–300 g) were killed by stunning and cervical dislocation. The thoracic aorta was rapidly removed into Krebs-Henseleit solution of the following composition (mM): NaCl 115, $NaHCO_3$ 25, $CaCl_2$ 2·5, KH_2PO_4 2·2, KCl 2, $MgSO_4$ 1·2, glucose 10, pH 7·4 and gassed with 5% CO_2 in O_2. The aorta was cleaned of adhering fat and connective tissue from the external surface and cut into a helical strip of approximate dimensions 15 × 4 mm. The strip was then denuded of endothelium by gently dragging the tissue, luminal surface down over moistened filter paper for 5–6 short strokes. This procedure was previously determined to be effective in removing endothelium since it abolished relaxant responses to acetylcholine (ACh) but had little effect on the relaxant properties of adenosine. Furthermore, histological studies based on the method of Griffith et al (1984) showed a practically complete removal of the endothelium by this procedure.

Release of ^{45}Ca from tissue

Aortic strips, freed of endothelium, were mounted under 0·8 g tension in calcium-free Krebs solution and contractile responses to NA (100 μM) were obtained. Digitonin and collagenase were added to the bathing medium to final concentrations of

0·005% and 0·1 U mL^{-1}, respectively, and allowed to incubate for 15 min. The solution was washed out with calcium-free Krebs and the tissue tested for responsiveness to NA (100 μM). When a contractile response was obtained the incubation in digitonin and collagenase was repeated. In most tissues the contractile responses to NA were abolished after one incubation and in all cases were abolished after two incubations.

The aortic strip was then mounted to cotton thread upon a glass tissue holder constructed from a single-bore microelectrode blank (Clark Electromedical Instruments Ltd) under approximately 0·8 g tension. The following solutions maintained at 37 °C throughout the experiment were employed in the succeeding stages (mM): solution 1: ATP 5·5, KCl 100, Tris 40, $MgCl_2$ 7·9, $CaCl_2$ 0·001, pH 6·8; solution 2: as solution 1 plus 9 μCi $^{45}CaCl_2$ mL^{-1}; solution 3: KCl 100, $MgCl_2$ 7·9, EGTA 1, Tris 40, pH 6·8.

Tissues treated with digitonin were incubated in a stirred 100 mL of solution 1 for 20 min. The tissue was then transferred to 2 mL of solution 2 for 60 min to load the tissue with ^{45}Ca and then washed in 8 changes of 20 mL solution 3 for 5 min each to remove non-sequestered label. The tissue was then incubated in 2·5 mL of this solution. 100 μL of the bathing solution were taken at 1 min intervals for scintillation counting and immediately replaced on each occasion with 100 μL of solution 3. A subsequent correction of counts min^{-1} was made to allow for the dilution of the medium. At various times throughout the experiment, NA (1 μM), adenosine (300 μM), inositol (10 μM) and IP_3 (5 μM) were included in the incubation medium. The concentrations stated are the estimated final concentrations in the incubation chamber.

The effect of adenosine on ^{45}Ca release

The effect of adenosine on ^{45}Ca release evoked by IP_3 was examined by performing two challenges, A and B, with IP_3 (5 μM). The challenges were performed 20 min apart with adenosine added 4 min before the second challenge. The protocol for this study is summarized in Fig. 1. The ratio of peak ^{45}Ca release B : A was used as an indication of the ability of the tissue to release ^{45}Ca.

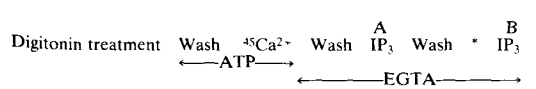

FIG. 1. Summary of the protocol of evoked release of $^{45}Ca^{2+}$ from digitonin-treated aortic preparations. A and B represent the periods of application of IP_3. The asterisk (*) indicates the point at which adenosine, adenosine deaminase or buffer were included in the bathing medium.

Adenosine effects on inositol phosphate turnover

Aortic strips, freed of endothelium, were mounted on a glass tissue holder under 0·8 g tension and incubated in Krebs solution at 37 °C for 1 h. The tissue and holder were transferred to vials containing 1·5 mL Krebs solution with 0·3 μM [^{3}H]myo-inositol (specific activity 16·3 Ci $mmol^{-1}$) and incubated for 1 h at 37 °C. The strips were then washed (3 × 20 mL Krebs) and transferred to 1 mL Krebs containing LiCl 10 mM, to inhibit inositol 1-phosphatase, for 10 min. 0·5 mL of Krebs, with or without drug, was added to yield the stated final concentration of NA (0 or 1 μM), adenosine (0 or 300 μM), dipyridamole (0 or 10 μM) or 8-phenyltheophylline (0 or 10 μM). The incubation was continued for 60 min and then halted by the addition of 0·9 mL chloroform–methanol (1 : 2) and vigorous vortexing. Water, 0·3 mL, and chloroform, 0·3 mL, were added. The tissue was then removed, lightly blotted and weighed. After further vortexing the aqueous phase was used for analysis of the inositol phosphates.

Separation of the water soluble [^{3}H]inositol phosphates was achieved by the method of Berridge et al (1982). Briefly, 0·7 mL of the aqueous phase was layered onto a Dowex 1-X8 column (formate form, 200–400 mesh). The columns were sequentially eluted with (a) 9 mL water; (b) 5 mL 5 mM sodium tetraborate/60 mM sodium formate; (c) 9 mL 5 mM sodium tetraborate/150 mM sodium formate; (d) 9 mL 0·1 M formic acid/0·75 M ammonium formate; (e) 5 mL 1 M formic acid. Each eluant was collected over one fraction and 0·4 mL aliquots of each fraction were taken for scintillation counting.

Materials

Chemicals were obtained from the following sources: Adenosine hemisulphate, disodium adenosine 5′-triphosphate (ATP), noradrenaline HCl, myo-inositol, inositol trisphosphate (IP_3), digitonin, Tris, EGTA, collagenase (EC 3.5.4.4) (Sigma Chemical Company); 8-phenyltheophylline (Calbiochem); dipyridamole (Karl Thomae); $^{45}CaCl_2$ [^{3}H]myo-inositol (Amersham radiochemicals); inositol-1,4,5-trisphosphate-D-[^{32}P] and D-[^{3}H]inositol-1-phosphate (New England Nuclear).

RESULTS

The efflux of ^{45}Ca into the bathing medium from digitonin-treated preparations of rat aorta is illustrated in Fig. 2. The background release of radioac-

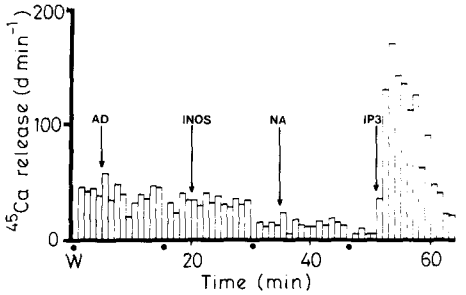

FIG. 2. The release of ^{45}Ca from a digitonin-treated aortic strip. The tissue was washed where indicated by the dot below the axis and then adenosine 300 μM (AD), inositol 10 μM (INOS), noradrenaline 1 μM (NA) or IP_3 5 μM were included 4 min later.

tivity may be due to label washing off membranes, to continuous basal release from organelles either as their normal activity or as a consequence of damage, or a combination of these factors. The baseline did, however, show a decay during the course of the experiment to approximately 10 d min^{-1}/aliquot.

Noradrenaline (1 μM), inositol (10 μM) and adenosine (300 μM) were added separately to the incubation medium with a wash between applications. None of these agents produced any demonstrable effect on release of label. IP_3 (5 μM), however, readily evoked a large release of ^{45}Ca into the incubation medium. The release was rapid, commencing within at least 1 min of the application of the agent and was sustained for approximately 15 min (Fig. 2).

Actions of adenosine on release of ^{45}Ca

Table 1 summarizes the release of ^{45}Ca obtained from digitonin-treated preparations in response to two consecutive challenges with IP_3 (5 μM). A release of ^{45}Ca was observed with both challenges, however the second challenge released somewhat less label than the first presumably due to partial depletion of intracellular stores. Results of release are expressed as the ratio of the two challenges with IP_3. Neither adenosine nor adenosine deaminase (to destroy any endogenous adenosine) produced any change in the ratio of the IP_3-induced calcium release.

Table 1. ^{45}Ca release from digitonin-treated aortic smooth muscle.

Agent added before B	Release ratio (B/A × 100)	n
Buffer	63·8 ± 8·6	6
Adenosine 300 μM	59·6 ± 5·1	6
Adenosine deaminase 5 u mL^{-1}	57·9 ± 14.2	4

The digitonin-treated tissue was challenged on two occasions, A and B. Adenosine, adenosine deaminase or buffer was included before the second (B) challenge.

There were no significant differences from the control values at $P < 0.05$ (t-test).

Effects on inositol phosphate content

The Dowex columns used for chromatography were characterized with authentic standards of inositol, IP_1 and IP_3. Greater than 98% of each standard applied to the column was recovered during the entire elution procedure and at least 95% of each standard was recovered within a single elution component. Chromatograms of the standards used are overlaid in Fig. 3 confirming that the protocol affords the resolution of these moieties into discrete fractions.

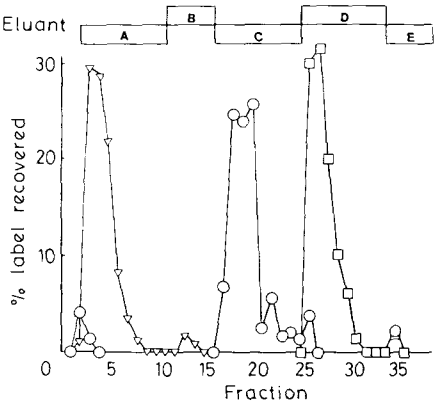

FIG. 3. The elution profile of inositol derivatives from a Dowex (1-X8) column. ▽ [^{3}H]inositol, ○ [^{3}H]IP_1 and □ [^{32}P]IP_3. The legend for the eluant corresponds to the legend for fractions in Table 2.

There was no significant change in the radiolabel content of fractions A or B with any of the experimental interventions used. The radiolabel content of fraction C, however, in which IP_1 was eluted from the columns was increased almost five-fold in response to noradrenaline (1 μM). Smaller, but statistically significant, increases in labelling of fractions D and E were also noted on application of noradrenaline (1 μM). These fractions have been associated with IP_3 and the higher phosphates of inositol, respectively (Berridge et al 1982) (Table 2).

The enhancement of labelling of the IP_1 fraction by NA was reduced by adenosine in an apparently concentration-dependent manner. Dipyridamole (10 μM) significantly potentiated the effect of a submaximal (50 μM) concentration of adenosine (P

Table 2. Phosphatidylinositol metabolite fractions from rat aorta.

Fractions	A	B	C	D	E
(a) Control (n = 5)	237·6 ± 65·2	9·8 ± 4·0	19·4 ± 4·0	6·02 ± 3·8	8·4 ± 2·9
(b) + NA 1 μM (n = 5)	189·4 ± 76·1	5·4 ± 2·2	*93·9 ± 24·5	*18·3 ± 7·6	*16·2 ± 4·6
(c) + NA 1 μM, aden 300 μM (n = 4)	207·8 ± 84·7	8·6 ± 3·8	*42·7 ± 13·2	10·7 ± 5·2	13·8 ± 7·2
(d) + NA 1 μM, aden 50 μM (n = 4)	218 ± 62·3	6·7 ± 4·0	76·3 ± 12·7	16·9 ± 8·0	11·7 ± 6·1
(e) + NA 1 μM, aden 300 μM, dipyr 10 μM (n = 4)	246·9 ± 71·6	6·1 ± 3·8	40·9 ± 20·8	14·8 ± 5·6	12·3 ± 4·2
(f) + NA 1 μM, aden 50 μM, dipyr 10 μM (n = 4)	232·7 ± 42·1	4·8 ± 2·9	*48·2 ± 8·6	20·8 ± 11·7	10·1 ± 5·2
(g) + NA 1 μM, aden 300 μM, 8PT 10 μM (n = 5)	216·9 ± 49	14·2 ± 8·1	*71·8 ± 13·7	14·0 ± 3·4	9·8 ± 4·8
(h) + aden 300 μM (n = 4)	251 ± 47·8	6·9 ± 1·5	24·7 ± 8·1	9·9 ± 5·8	7·5 ± 3·6
(j) + NA 1 μM, 8PT 10 μM (n = 4)	222·1 ± 58·1	8·3 ± 5·9	87·9 ± 19·9	21·7 ± 8·2	10 ± 5·2

NA = noradrenaline; aden = adenosine; dipyr = dipyridamole; 8PT = 8-phenyltheophylline.
Values are mean ± s.e.m. in counts min^{-1} mg $tissue^{-1}$.
Comparison between results was performed using a two-tailed *t*-test between the following rows: (b) and (h) with (a); (c), (d) and (j) with (b); (f) with (d); (g) with (c).
* Significant difference at $P < 0·05$. Fraction C includes inositol-1-phosphate.

< 0·05) indicating that the site of action of adenosine in this respect is likely to be extracellular. Furthermore, 8-phenyltheophylline (10 μM) reversed the adenosine-induced reduction in labelling of IP_1.

DISCUSSION

The sarcoplasmic reticulum (SR) of vascular smooth muscle is believed to store sufficient calcium to provide contraction when the ion is released (Bond et al 1984; Somlyo & Somlyo 1975; Garfield & Somlyo 1985). The results presented here clearly show that a specific release of ^{45}Ca was achieved on addition of IP_3 to preparations whose plasma membranes had been made hyperpermeable by digitonin and collagenase, a treatment which is thought not to affect intracellular membranes lacking cholesterol. Myo-inositol, adenosine or NA did not evoke any release of ^{45}Ca from the preparation. In addition, whilst the second challenge of IP_3 typically released approximately 60% of the label compared with the first challenge, adenosine did not affect this ratio. Since adenosine does not affect the sequestration of ^{45}Ca into the SR of this tissue (unpublished observations) the purine does not seem to have any direct effects on this organelle.

It has been demonstrated here that noradrenaline increases the tissue content of IP_1 during blockade of inositol 1-phosphatase by lithium (see also Legan et al 1985). Activation of α_1-adrenoceptors also increases inositol metabolism in rat vas deferens and tail artery (Fox et al 1985) as well as in the aorta. Campbell et al (1985) have correlated (indirectly) the hydrolysis of phosphatidylinositol with calcium flux and ultimately contraction in the rabbit aorta. Certainly IP_3 is associated with ^{45}Ca release from intracellular sites as demonstrated here (see also Yamamoto & van Breemen 1985) and in a highly sophisticated experiment Somlyo et al (1985) showed that IP_3 not only induced calcium release in digitonin-treated smooth muscle fibres, but that this was quantitatively sufficient to induce an increase in tone in the preparation. Phosphorylation of inositol is believed to occur subsequent to incorporation of the sugar into phosphatidylinositol (Hirasawa & Nishizuka 1985), consequently an adenosine-induced reduction in phosphatidylinositol turnover is inferred from the results presented here.

The adenosine receptor mediating this response is probably located within the plasma membrane since its effects could be presented by the purine receptor antagonist 8-phenyltheophylline, furthermore the nucleoside uptake inhibitor dipyridamole increased the effect of adenosine at intermediate concentrations of the purine.

The ability of adenosine to reduce tissue levels of biologically relevant phosphates of inositol will in turn therefore reduce the quantity of calcium released from intracellular stores and the magnitude of agonist-induced contraction. We therefore propose that this phenomenon may be involved in the mechanism by which adenosine produces vasorelaxation. We have demonstrated (Long & Stone 1985) that adenosine is of lesser potency when acting versus contractions believed to be based upon wholly extracellular calcium. If IP_3 does indeed modulate intracellular calcium release an adenosine-induced reduction of IP_3 production may account for this observation on calcium sources. More recently, Kuno & Gardner (1987) have described activation of plasma membrane calcium channels by IP_3 which

naturally implies that increased free cytosolic calcium from enhanced PI turnover may not be of solely intracellular origin.

Adenosine's actions on second messenger function has been well-described with regard to adenylate cyclase where it has been noted that either an increase or a decrease in cAMP content may occur which is dependent upon the adenosine receptor subtype activated (van Calker et al 1979). Such an action might also occur with phosphatidylinositol metabolism, since whilst Rubio (1986) showed that adenosine enhanced the decrease in phosphatidylinositol monophosphate and bisphosphate in oxytocin-stimulated rat adipocytes, an apparently opposite action was demonstrated by Curnish et al (1986) in the frog sympathetic ganglion, in which adenosine inhibited phosphatidylinositol turnover. Such results imply that adenosine may therefore exhibit actions upon multiple second messenger sytems. Hollingsworth & Daly (1985) have discussed a possible interrelationship between cAMP accumulation and phosphatidylinositol turnover since diacylglycerols generated after hydrolysis of the inositol group stimulated protein kinase C which may lead to an enhanced responsiveness of adenylate cyclase upon stimulation. An interrelationship between cAMP and cGMP has already been speculated upon by Terasaki & Appleman (1975); the inclusion of a further level of modulation of second messenger function would considerably complicate the understanding of function and action of these moieties.

Acknowledgements

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