Cross-talk between muscarinic- and adenosine-receptor signalling in the regulation of cytosolic free Ca^{2+} and insulin secretion

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The effects of A1-adenosine-receptor occupation on Ca^{2+} handling in the insulin-secreting RINm5F cell line were investigated. The selective A1-agonist *N*⁶-cyclopentyladenosine (CPA) had no effect itself on the cytosolic free Ca^{2+} concentration in cells loaded with Fura 2. However, CPA (1) attenuated the rise due to activation of voltage-gated Ca^{2+} channels with Bay K 8644, and (2) caused a secondary increase (EC_{50} approx. 300 nM) if added after the primary Ca^{2+} -mobilizing agonists vasopressin or carbamoylcholine (carbachol). Prior addition of CPA (10 μM) also potentiated (by approx. 20%) the subsequent Ca^{2+} peak due to maximal (100 μM) carbachol, but did not alter the EC_{50} of the carbachol response. Detailed analysis of the secondary rise in Ca^{2+} revealed further features. First, it was due to mobilization from intracellular stores, since it persisted in the absence of extracellular Ca^{2+} . Second, it was associated with a rapid (5–15 s) increase in phospholipase C (PLC) activity, as measured by h.p.l.c. analysis of $\text{Ins}(1,4,5)\text{P}_3$. This increase was only apparent

after prior stimulation with carbachol. Third, and unlike the response to carbachol, it was mediated by a pertussis-toxin-sensitive G-protein. Fourth, it was not secondary to a decrease in cyclic AMP. Fifth, it was absolutely dependent on continued occupation of the primary receptor, since it was abolished if carbachol was displaced with the antagonist atropine. This implies a dynamic cross-talk between the two receptor coupling systems, rather than covalent modification as a result of the prior activation of PLC. Sixth, it was not associated with any desensitization of the ability of CPA to inhibit forskolin-stimulated adenylate cyclase activity. Glyceraldehyde (10 mM)-induced insulin secretion was also potentially inhibited by CPA > 10 nM, but the secretory response to 100 μM carbachol was unaffected up to 10 μM . The results suggest that, *in vivo*, adenosine would inhibit secretion due to carbohydrate nutrients much more effectively than that due to stimuli which activate PLC.

INTRODUCTION

Insulin secretion from the pancreatic β -cell is modulated, both positively and negatively, by a wide variety of physiological regulators [1]. The most important of the activators are nutrient secretagogues, such as glucose, which depolarize the plasma membrane and thereby raise the cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) as a consequence of the activation of voltage-dependent Ca^{2+} channels [2,3]. Other regulators such as neurotransmitters, hormones and local mediators bind to specific cell-surface receptors linked, via heterotrimeric G-proteins, to a variety of intracellular effector systems [3,4]. For example, the occupation of muscarinic receptors is coupled, presumably via the G-protein Gq [5], to the stimulation of phospholipase C (PLC) and resultant hydrolysis of PtdInsP_2 [6,7]. Two second messengers are thereby produced: $\text{Ins}(1,4,5)\text{P}_3$, which releases Ca^{2+} from intracellular stores, and diacylglycerol, which activates protein kinase C (PKC) [8,9]. Glucagon, on the other hand, stimulates secretion via a rise in cyclic AMP, a second messenger generated as a consequence of the activation of adenylate cyclase by the G-protein Gs [10]. Finally, insulin release is inhibited by a class of agents which include adrenaline (acting via α_2 -adrenergic receptors) as well as somatostatin and galanin. Although the receptors for these agents are coupled via the pertussis-toxin (PT)-sensitive G-protein G_i , to inhibition of adenylate cyclase, it is apparent that the resultant decrease in cyclic AMP levels is not a major factor in the inhibition of secretion [11–14]. Other responses,

such as hyperpolarization of the plasma membrane due to the activation of K^+ channels, or closure of voltage-dependent Ca^{2+} channels, are more likely to be implicated in the inhibition of the secretory response [14–19]. In addition, there is evidence for an inhibitory site which is distal to second messenger generation, and might be at the very last stages of the exocytotic process [12–14,17,20].

The local mediator adenosine, acting most probably via the A1-receptor subtype, is also known to inhibit insulin secretion and islet-cell adenylate cyclase activity [21–23]. However, in contrast with the agonists listed above, very little is known of its effects on Ca^{2+} handling in insulin-secreting cells. In neuronal cell lines, occupation of A1 receptors results in the closure of voltage-gated Ca^{2+} channels [24], whereas in the pituitary GH3 cell line [25] and mouse cortical slices [26] it inhibits agonist-stimulated increases in $\text{Ins}(1,4,5)\text{P}_3$ and presumably Ca^{2+} mobilization. On the other hand, occupation of A1 receptors directly stimulates PtdInsP_2 hydrolysis in cell lines derived from renal tissue [27], mast cells [28] and smooth muscle [29]. A third type of response has been reported in guinea-pig cortical slices [30,31] and a thyroid cell line [32,33] in which adenosine analogues exerted no effect by themselves, but potentiated the $\text{Ins}(1,4,5)\text{P}_3$ response due to an agonist capable of stimulating PtdInsP_2 hydrolysis. In the present study we demonstrate that A1 agonists inhibit voltage-dependent Ca^{2+} influx in the insulin-secreting cell line RINm5F, but can also increase $[\text{Ca}^{2+}]_i$. The latter response is totally dependent on the continued presence of a primary Ca^{2+} -

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; PT, pertussis toxin; KRB, Krebs–Ringer bicarbonate; CPA, *N*⁶-cyclopentyladenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; NECA, 5'-*N*-ethylcarboxamidoadenosine; PIA, phenylisopropyladenosine; CGS-21680, 2-*p*-(2-carboxyethyl)phenylamino-5'-*N*-ethylcarboxamidoadenosine hydrochloride; G-protein, GTP-binding proteins; PLC, phospholipase C; PKC, protein kinase C; dbcAMP, dibutyryl cyclic AMP; PMA, phorbol 12-myristate 13-acetate.

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mobilizing stimulus, and represents a dynamic cross-talk between the two classes of receptor.

EXPERIMENTAL

Cell culture

RINm5F cells were cultured in 75 cm² flasks with 20 ml of RPMI 1640 medium containing 24 mM NaHCO₃, 10% (v/v) fetal-calf serum, 50 i.u. of penicillin/ml and 50 µg of streptomycin/ml. Individual flasks were flushed with 5% CO₂/air, tightly stoppered and incubated at 37 °C. Media was changed every 2–3 days. For harvesting, the cells were detached with a phosphate-buffered saline solution containing 0.025% (w/v) trypsin and 0.2 mM EDTA. The cells [(30–50) × 10⁶] were washed and resuspended in RPMI 1640 supplemented with 14 mM NaHCO₃, 10 mM Hepes, 1% (w/v) newborn-calf serum, 100 i.u. of penicillin/ml and 100 µg of streptomycin/ml. They were then maintained at 37 °C in spinner culture for 3 h [6]. For some experiments, PT (100 ng/ml final concn.) was included in the spinners.

Fura 2 measurements

Cells were maintained in spinner culture as described above at a concentration of 10⁶/ml. For the last ½ h, the medium was supplemented with 1 µM Fura 2 acetoxymethyl ester (AM; final concn.) added from a 1 mM stock in dimethyl sulphoxide. The cells were then washed twice with spinner medium to remove extracellular Fura 2, and resuspended at 5 × 10⁶/ml and distributed at 0.4 ml portions to be maintained at room temperature until required. Immediately before use the spinner medium was removed, and the cells were resuspended in 2 ml of Krebs–Ringer bicarbonate (KRB) buffer (pH 7.4) at 37 °C containing 10 mM Hepes, 5 mM NaHCO₃, 2.8 mM glucose and 1 mM CaCl₂. Sulphinpyrazone (200 µM final concn. from a 200 mM stock in dimethyl sulphoxide) was also added to minimize Fura 2 leakage. The cells were transferred to a quartz cuvette and placed in an Hitachi F-4010 fluorimeter, where they were maintained at 37 °C under continuous stirring. The fluorescence was monitored by using excitation and emission wavelengths of 340 and 505 nm respectively. Before experimental additions, fluorescence due to extracellular Fura 2 was estimated as that portion of the signal which was rapidly quenched by the addition of MnCl₂ (50 µM). The latter was then chelated with diethylenetriaminepenta-acetic acid (100 µM). The calibration of [Ca²⁺]_i was performed by lysing the cells with 0.04% (v/v) Triton X-100, and determining the maximal and minimal fluorescence in the presence of EGTA (4 mM) and CaCl₂ (5 mM) respectively. Values for [Ca²⁺]_i were then calculated by using the published formula [34]. All fluorescence tracers are typical of at least three separate experiments.

Ins(1,4,5)P₃ measurements

For analysis of inositol phosphates, the cultured cells were supplemented with [³H]inositol (10 µCi/ml) during both the last 48 h before harvesting and the 3 h spinner culture. Cells were subsequently washed and resuspended (10⁶ cells/0.5 ml) in KRB buffer as described above, except for the addition of 0.5% BSA. Incubations and extraction of inositol phosphates and their preparation for h.p.l.c. were exactly as reported previously [6]. H.p.l.c. analysis was performed on a Whatman Partisphere PAC column (12.5 cm × 0.4 cm) eluted with ammonium phosphate (pH 3.8). The exact gradient comprised an initial 5 min wash with water, followed by 0–0.1 mM ammonium phosphate over 35 min, and 0.1 to 1.0 mM over 55 min. Under these conditions Ins(1,4,5)P₃ was routinely eluted at around 60 min, and Ins(1,3,4)P₃ 2 min earlier.

Figure 1 Effect of the A1-adenosine-receptor agonist CPA (10 µM) on [Ca²⁺]_i in RINm5F cells

Cells pre-loaded for 30 min with 1 µM Fura 2 AM were washed and resuspended at 2.0 × 10⁶ cells/2 ml in KRB buffer. They were maintained at 37 °C under continuous stirring in a cuvette placed in a Hitachi F-4010 fluorimeter. Fluorescence was maintained by using excitation and emission wavelengths of 340 and 505 nm respectively. Response time was 2 s. For further details see the Experimental section. Gaps in the traces are due to opening of the compartment lid to make additions. These additions, marked by the vertical arrows, were: BAY K, BAY K8644 (1 µM); carb, carbachol (100 µM); and CPA (10 µM).

Cyclic AMP measurements

For estimation of cyclic AMP, unlabelled cells were resuspended and preincubated in KRB under the same conditions as described for the inositol phosphate studies. Incubations were for 5 min and were terminated by addition of 0.1 ml of trichloroacetic acid (100%, v/v). The soluble extract was then washed with 3 × 4 ml of diethyl ether, and cyclic AMP content was measured by radioimmunoassay with a commercial kit.

Insulin secretion

Insulin release was measured in static incubations by using unlabelled cells in KRB buffer plus 0.5% BSA. Cells were suspended at 10⁶/ml in 1 ml samples and preincubated for 15 min at 37 °C. After centrifugation, 0.8 ml of the supernatant was removed for estimation of basal insulin secretion. Experimental additions (0.8 ml) were made from stock solutions concentrated 1.25-fold in pre-warmed KRB [18]. After incubation

Figure 2 Dose-dependences of the interaction between carbachol and CPA effects on $[Ca^{2+}]_i$

RINm5F cells were loaded with Fura 2, and $[Ca^{2+}]_i$ was measured as described in the legend to Figure 1. (a) Cells were stimulated with various doses of carbachol after pretreatment (●) or not (○) with 10 μ M CPA. Results are presented as the increments above baseline ($n = 4$). (b) Various doses of CPA were added 1.5 min after 100 μ M carbachol. Results ($n = 4$) are presented as percentages of the incremental response to 10 μ M CPA, which averaged 45 ± 7 nM.

for 15 min at 37 °C, the tubes were chilled to 4 °C, centrifuged, and a sample of the supernatant was stored for insulin measurement by radioimmunoassay.

Results are presented as means $\pm$ S.E.M. and statistical significance was determined by Student's t test.

Materials

Tissue-culture media and supplies were obtained from Flow Laboratories, Sydney, N.S.W., Australia, except for plasticware, which was from Becton Dickinson and Co., Lincoln Park, NJ, U.S.A. N^6 -Cyclopentyladenosine (CPA), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) and 2- p -(2-carboxyethyl)phenylamino-5'- N -ethylcarboxamidoadenosine hydrochloride (CGS-21680) were from Research Biochemicals Inc., Natick, MA, U.S.A. [3H]Inositol and 3-[^{125}I]iodotyrosyl-A14-insulin were supplied by Amersham Australia Pty. Ltd., Sydney, N.S.W., Australia. Cyclic AMP radioimmunoassay kits were obtained from Du Pont (Australia) Ltd., Sydney, N.S.W., Australia. Fura 2 AM was purchased from Molecular Probes, Eugene, OR, U.S.A. Insulin radioimmunoassay reagents were supplied as follows: rat insulin standard from Novo, Copenhagen, Denmark; guinea-pig anti-(pig insulin) serum from Linco Research, St. Louis, MO, U.S.A.; and sheep anti-(guinea-pig Ig) from Silenus Laboratories, Melbourne, Victoria, Australia. All other biochemicals and specialized reagents were from Sigma Chemical Co., St. Louis, MO, U.S.A.

RESULTS

Occupation of adenosine receptors on RINm5F cells, by the selective A1-agonist CPA, led to one of three effects on $[Ca^{2+}]_i$, depending on the prevailing conditions (Figure 1). Firstly, a rapid fall in $[Ca^{2+}]_i$ was observed if CPA was added after BAY

Figure 3 Pharmacological characterization of the effect of CPA on $[Ca^{2+}]_i$

RINm5F cells were loaded with Fura 2, and $[Ca^{2+}]_i$ was measured as described in the legend to Figure 1. Vertical arrows mark the addition of: carb, carbachol (100 μ M); or CPA, DPCPX, CGS-21680 or NECA at the indicated concentrations (μ M).

Figure 4 Source and G-protein-dependence of the effect of CPA on $[Ca^{2+}]_i$

RINm5F cells were loaded with Fura 2 after pretreatment in the absence (a and b) or presence (c) of PT (100 ng/ml). $[Ca^{2+}]_i$ was measured as described in the legend to Figure 1. Vertical arrows mark the addition of: carb, carbachol (100 μ M); CPA (10 μ M); EGTA (4 mM).

K8644 (Figure 1a). The latter, a pharmacological activator of voltage-dependent Ca^{2+} channels, would otherwise have maintained $[Ca^{2+}]_i$ at an elevated plateau (results not shown). Therefore the inhibitory effect of CPA is likely to be due to blockade of those voltage-gated channels. Secondly, in the absence of any other additions, CPA did not alter the resting $[Ca^{2+}]_i$ (Figure 1b). Thirdly, CPA initiated a secondary increase in $[Ca^{2+}]_i$ if added after carbachol, a muscarinic receptor agonist (Figure 1c). This response was also apparent in another form: the peak rise in $[Ca^{2+}]_i$ due to carbachol was increased if CPA was added beforehand, even though CPA exerted no direct effect on $[Ca^{2+}]_i$ itself (cf. Figures 1b and 1c). When results were averaged from 15 different cell preparations, this potentiation amounted to an approx. 20% increase in the peak height (117 ± 7 versus 142 ± 7 nM in control and CPA-pretreated respectively; $P < 0.02$). This response was further examined by measuring the $[Ca^{2+}]_i$ peak heights to a range of carbachol concentrations in either the absence or presence of 10 μ M CPA (Figure 2a). The effect of CPA was clearly to increase the maximal capacity of the response, without obviously altering the EC_{50} of carbachol for Ca^{2+} mobilization.

The CPA effect was further examined in order to confirm whether an A1-like adenosine receptor was implicated. From

Figure 5 Effect of carbachol and CPA on $Ins(1,4,5)P_3$ generation in RINm5F cells

Cells were pre-labelled with $[^3H]$ inositol for 48 h, washed and resuspended at 10^6 cells/0.5 ml in KRB buffer. After extraction, inositol phosphates were separated by anion-exchange h.p.l.c. For further details see the Experimental section. Incubations were performed after addition (●) or not (○) of 100 μ M carbachol at zero time. At the point marked by the arrow 10 μ M CPA was added (■) or not (●). Results ($n = 5-6$) are expressed as a percentage of the zero-time control, which averaged 45.2 ± 7.3 c.p.m.

Figure 2(b) the EC_{50} for the secondary Ca^{2+} response was estimated as approx. 300 nM, broadly consistent with an A1-receptor sub-type. In addition, the effect of 10 μ M CPA was abolished after pretreatment of the cells with a 100-fold lower concentration of the highly selective A1-antagonist, DPCPX (Figures 3a and 3b). Under these conditions the rise in $[Ca^{2+}]_i$ due to carbachol was unaffected. Moreover, the A2a agonist CGS-21680 (1 μ M) was unable to substitute for CPA (Figure 3c). Similarly 5'-N-ethylcarboxamidoadenosine (NECA), a non-specific agonist with relatively low affinity for both A1 and A2 receptors, was also without effect when used at 250 nM (Figure 3d). In contrast, an identical concentration of CPA did cause a secondary rise in $[Ca^{2+}]_i$, even when added after NECA (Figure 3d).

The potentiation of the carbachol-induced rise in $[Ca^{2+}]_i$ by CPA could be explained by stimulated Ca^{2+} influx from the extracellular space, enhanced mobilization from intracellular stores, or both. In order to differentiate between these alternatives, EGTA was used to chelate extracellular Ca^{2+} immediately before the addition of CPA (Figure 4). Although slightly diminished in the presence (Figure 4b) versus absence (Figure 4a) of EGTA, a rise in $[Ca^{2+}]_i$ was still obtained. This indicates that, as previously described for carbachol [6], release of Ca^{2+} from intracellular stores was the predominant response. However, the post-receptor events initiated by the two agonists were not identical, since in PT-pretreated cells the CPA response was abolished, whereas that of carbachol was unaffected (Figure 4c). This demonstrates that the G-protein mediating the effects of A1-receptor activation is a member of the PT-sensitive G_i , or G_o family, as opposed to that (most probably G_q) which transduces the muscarinic response.

Since the best-defined mechanism by which receptor-binding agonists mobilize Ca^{2+} is via the generation of $Ins(1,4,5)P_3$, we next checked whether CPA potentiated $PtdInsP_2$ hydrolysis in response to carbachol. Muscarinic stimulation of RINm5F cells resulted in a rapid (5 s) increase in $Ins(1,4,5)P_3$ which was significantly different ($P < 0.005$) from the zero-time control (Figure 5). Consistent with earlier studies, in which it was shown that the carbachol response was maximal at 5–10 s [6,35], $Ins(1,4,5)P_3$ thereafter declined, until at 90 s it was no longer significantly elevated relative to unstimulated cells measured at either 0 or 105 s (both $P > 0.05$). However, when 10 μ M CPA

Figure 6 Further characterization of the cross-talk responses between CPA and a primary Ca^{2+} -mobilizing stimulus

RINm5F cells were loaded with Fura 2 and $[\text{Ca}^{2+}]_i$ was measured as described in the legend to Figure 1. Vertical arrows mark the addition of: carb, carbachol ($100 \mu\text{M}$); vaso, vasopressin ($1 \mu\text{M}$); atrop, atropine ($10 \mu\text{M}$); dbcAMP ($100 \mu\text{M}$); and CPA ($10 \mu\text{M}$).

Figure 7 Effect of PKC depletion on the $[\text{Ca}^{2+}]_i$ responses to CPA, carbachol and vasopressin

RINm5F cells were pretreated for 24 h in tissue culture in either the absence (a and b) or presence (c and d) of 100 nM PMA. Cells were loaded with Fura 2 and $[\text{Ca}^{2+}]_i$ was measured as described in the legend to Figure 1. Vertical arrows mark the addition of: carb, carbachol ($100 \mu\text{M}$); CPA ($10 \mu\text{M}$); vaso, vasopressin ($1 \mu\text{M}$); PMA (100 nM).

was added at the 90 s time point, $\text{Ins}(1,4,5)\text{P}_3$ was significantly re-elevated within a further 5 s as compared with the unstimulated controls ($P < 0.05$ versus either 0 or 105 s). At 15 s this increase was even more pronounced ($P < 0.01$ versus 105 s control, $P < 0.05$ versus 90 s carbachol alone) and approximately equivalent to the earlier peak. However, addition of $10 \mu\text{M}$ CPA to cells which had been incubated for 90 s in the absence of carbachol did not significantly raise $\text{Ins}(1,4,5)\text{P}_3$ ($106 \pm 11\%$ of zero-time control with CPA, versus 100 ± 6.7 or $107 \pm 6.0\%$ for control: at 0 or 105 s respectively; $n = 4-5$).

Possible mechanisms underlying this potentiation were examined in a further series of experiments performed with Fura 2-loaded cells (Figure 6). It was firstly established that the secondary mobilization of Ca^{2+} (Figure 6a) was not related to the ability of CPA to inhibit adenylate cyclase, since the response was still apparent in the presence of an excess of cyclic AMP, added exogenously in the form of $100 \mu\text{M}$ dibutyl cyclic AMP (dbcAMP) (Figure 6b). Similar results were obtained if the preincubation time was extended to 10 min, or if $100 \mu\text{M}$ 8-bromocyclic AMP was used instead. It was next demonstrated

Figure 8 Inhibition of 50 μM forskolin-stimulated cyclic AMP accumulation by CPA in the absence (a) or presence (b) of carbachol

RINm5F cells were incubated at 10^6 cells/ml of KRB for 5 min. Cyclic AMP was measured in cell extracts by radioimmunoassay. Results are expressed as a percentage of the incremental stimulation by 50 μM forskolin in either the absence or presence of 100 μM carbachol. This concentration of forskolin gave an average 6–7-fold increase in cyclic AMP over the basal levels of 15 (a) or 12 (b) pmol/ 10^6 cells. Results are the means of 7–9 (a) or 8–9 (b) individual observations.

that vasopressin, as well as carbachol, could fulfil the requirement of a primary agonist (Figure 6c). However, when the muscarinic antagonist atropine (10 μM) was added 15 s before addition of CPA (in order to displace carbachol from its receptor), the secondary response was abolished (cf. Figures 6a and 6d). This demonstrates that continued occupation of the muscarinic receptor was necessary, and suggests that the secondary response was due to cross-talk between the two receptor classes and was not simply a function of prior Ca^{2+} mobilization or activation of PKC. The latter conclusion was supported by results (not shown) using thapsigargin, a tumour-promoting alkaloid which mobilizes Ca^{2+} from intracellular stores [36], and the phorbol ester phorbol 12-myristate 13-acetate (PMA), which activates PKC [9]. When thapsigargin was used to generate a $[Ca^{2+}]_i$ response similar to that of carbachol, a following addition of CPA was without effect. Acute treatment with PMA, in either the absence or presence of thapsigargin, was also insufficient to allow CPA to mobilize Ca^{2+} .

However, the possibility remained that phosphorylation by PKC, rapidly reversed by phosphatases after removal of carbachol, might be important for the cross-talk response. The role of PKC was addressed more directly in the next series of experiments, by using Fura-2-loaded cells (Figure 7). As shown in Figure 7(a), addition of vasopressin after both carbachol and CPA resulted in a small increase in $[Ca^{2+}]_i$, which was less pronounced than the rise due to vasopressin alone (cf. Figure 6c, for example). This form of down-regulation due to the earlier additions is well described and has been postulated to involve activation of PKC [37]. This possibility is supported by the fact that the residual response to vasopressin was completely abolished by the immediate prior addition of PMA (Figure 7b).

Figure 9 Dose-dependence of the effects of CPA on insulin secretion

RINm5F cells were incubated at 10^6 cells/ml of KRB for 10 min at 37 $^{\circ}C$. Insulin released into the medium was measured by radioimmunoassay. For further details see the Experimental section. For (a), insulin release was stimulated with either glyceraldehyde (10 mM) or ionomycin (iono; 1 μM) plus dbcAMP (100 μM). Results are 3–4 replicates from a single experiment representative of three. Basal insulin secretion was 2.4 ng/10 min per 10^6 cells. For (b) insulin release was stimulated with 100 μM carbachol. All values ($n = 7$ –8) were significantly ($P < 0.05$) greater than basal, which was 0.69 ± 0.08 ng/10 min per 10^6 cells.

Similar experiments were performed by using cells which had been pretreated for 24 h with 100 nM PMA, conditions which have previously been shown to deplete total PKC activity by up to 96% in RINm5F cells [38]. In this instance, the final response to vasopressin was much larger than in the non-depleted cells (cf. Figures 7a and 7c) and was no longer abolished by the immediate prior addition of PMA (cf. Figures 7b and 7d). The latter finding clearly shows that PKC could no longer be activated in the depleted cells. Importantly, CPA was equally effective in raising $[Ca^{2+}]_i$ in both the PMA-pretreated and control cells, suggesting that this response was independent of the presence of functional PKC activity.

One possible explanation for the results described above is that carbachol disrupts the coupling between adenylate cyclase and the PT-sensitive G-protein, and promotes instead an interaction between the latter and PLC. The ability of CPA to inhibit forskolin-stimulated adenylate cyclase activity was therefore measured in the absence or presence of carbachol (Figure 8). Stimulated cyclic AMP production was maximally inhibited by 67% ($P < 0.001$) in the presence of 1 μM CPA; half-maximal inhibition was obtained at approx. 20 nM CPA (Figure 8a). As shown in Figure 8(b), the ability of either maximal (1 μM) or sub-maximal (10 nM) concentrations of CPA to inhibit adenylate cyclase was not altered in the presence of carbachol (both $P > 0.05$ versus the corresponding CPA concentration without carbachol).

Since CPA has already been shown to inhibit Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Figure 1a), it would be expected that glyceraldehyde-stimulated secretion would also be inhibited. As shown in Figure 9(a), this was indeed the case, with a significant ($P < 0.01$) attenuation being apparent with as

little as 10 nM CPA. However, a site independent of Ca^{2+} influx is probably also involved, since 10 nM CPA also inhibited ($P < 0.05$) insulin release due to the pharmacological activators dbcAMP and ionomycin, a Ca^{2+} ionophore (Figure 9a). Under these conditions the inhibitory effect could not be mediated by alterations in $[\text{Ca}^{2+}]_i$ and cyclic AMP, and therefore reflects the interaction with a distal site [12–14,17,20]. In marked contrast with the above findings, CPA, over the range 1 nM–10 μM did not significantly alter the release of insulin due to 100 μM carbachol (Figure 9b). This implies that the distal inhibitory site is not activated in the presence of carbachol.

DISCUSSION

The presence of functional A1-adenosine receptors on pancreatic β -cells has previously been inferred from the inhibition of both adenylate cyclase activity and glucose-stimulated insulin release by adenosine and adenosine analogues such as phenylisopropyladenosine (L-PIA) [21–23]. In addition, the latter has been shown to attenuate slightly the unidirectional influx of $^{45}\text{Ca}^{2+}$ due to glucose stimulation [22]. These findings have been extended in the present study to the RINm5F cell line, which secretes insulin in response to glyceraldehyde via mechanisms which are very similar to those by which glucose activates native β cells [3]. However, the main focus of the current investigation was the description and characterization of a stimulatory effect on Ca^{2+} mobilization brought about by occupation of adenosine receptors.

Although a rigorous receptor classification was not the object of this study, there is good evidence for the involvement of an A1, rather than A2, receptor subclass. Thus the effect of CPA on Ca^{2+} mobilization in RINm5F cells was completely abolished by a 100-fold lower concentration of the selective A1-antagonist DPCPX, and it was not reproduced by the A2a agonist CGS-21680 [39,40]. Moreover NECA, as opposed to CPA, was ineffective at 250 nM. Although the former is non-selective for the two major forms of adenosine receptor, it is known to display a lower affinity than CPA for A1-receptors [39,40]. However, the response described here is slightly atypical, in that the EC_{50} of 300 nM is 5–10-fold higher than that required for inhibition of adenylate cyclase in either RINm5F cells (Figure 8) or fat cell membranes [41]. It is also 10-fold higher than that needed for the direct stimulation of inositol phosphate generation in the DDTMF-2 hamster smooth-muscle cell line [29]. This might indicate the presence of a different receptor isoform in RINm5F cells.

Depending on tissue and species, A1-agonists have been shown to exert divergent effects on PtdInsP_2 hydrolysis and/or Ca^{2+} mobilization: direct stimulation in renal, mast and smooth-muscle cells [27–29]; inhibition in GH3 cells and mouse brain cortical slices [25,26]; and potentiation of a primary agonist in guinea-pig cortical slices [30,31] and FRTL-5 thyroid cells [32,33]. The findings of the present study clearly place RINm5F cells in the last category, although there are differences as compared with the previous reports. Firstly, the response in guinea-pig cortex was mediated by a receptor which showed a pharmacological profile which was atypical of either A1 or A2 receptors and, additionally, the primary agonist had to be histamine: other Ca^{2+} -mobilizing stimuli such as carbachol and α_1 -adrenergic agonists were ineffective [30]. In the present study both carbachol and vasopressin sufficed, and the adenosine receptor was clearly better described as A1 rather than A2. With the thyroid cells, the A1-agonist PIA shifted the thyrotropin dose-response curve for Ca^{2+} mobilization nearly 2 orders of magnitude to the left [32].

In RINm5F cells the effect of CPA was clearly to increase the capacity of carbachol to raise $[\text{Ca}^{2+}]_i$ without altering the EC_{50} . However in both studies, a PT-sensitive G-protein was involved. Candidates in the RINm5F cell would be $\text{G}_i\alpha 1$, $\text{G}_i\alpha 2$, $\text{G}_o\alpha 1$ and $\text{G}_o\alpha 2$ [42].

The potentiation of Ca^{2+} mobilization by A1 agonists is best detailed for those cell types in which the A1 receptor is itself linked to the stimulation of PtdInsP_2 hydrolysis [27–29]. Less well characterized is the response which is wholly dependent on a primary agonist, and a number of novel findings concerning this type of response are reported in the present study. Firstly we have formally demonstrated that Ca^{2+} mobilization from an intracellular Ca^{2+} store really was involved (as opposed to Ca^{2+} influx), because the increase in $[\text{Ca}^{2+}]_i$ was still apparent when extracellular Ca^{2+} was chelated with EGTA. However, this does not preclude (voltage-independent) Ca^{2+} influx playing some role in the response. Secondly, PtdInsP_2 hydrolysis was implicated as the cause of the Ca^{2+} mobilization by the demonstrated rapid increase in $\text{Ins}(1,4,5)\text{P}_3$. Strictly speaking, the previously reported increases in total inositol phosphates, measured after prolonged stimulation (30–60 min) in the presence of LiCl, could be explained by the generation of $\text{Ins}(1,4,5)\text{P}_3$ (or non-functional inositol phosphates) secondary to a rise in $[\text{Ca}^{2+}]_i$ brought about by some other mechanism [30,31,33]. It should also be noted that, although our results are almost certainly explained by a potentiation of PtdInsP_2 hydrolysis, we cannot formally exclude an inhibition of $\text{Ins}(1,4,5)\text{P}_3$ degradation.

Thirdly, it was shown that the secondary Ca^{2+} response was still apparent in the presence of exogenous dbcAMP. This rules out an otherwise credible mechanism whereby CPA, by decreasing adenylate cyclase activity, might relieve a tonic inhibition of PtdInsP_2 hydrolysis due to cyclic AMP-dependent phosphorylation [43]. However, other phosphorylation reactions are well known to be involved in mediating cross-talk between receptors linked to activation of PLC and those which modulate adenylate cyclase [37]. For example, PKC has been demonstrated to phosphorylate both α - and β -adrenergic receptors, as well as the α subunit of G_i [37]. However, involvement of PKC is precluded by the finding that CPA still induced a secondary rise in $[\text{Ca}^{2+}]_i$ in PKC-depleted cells (Figure 7).

The fourth novel finding of the present study was the absolute requirement for continued occupation by the primary agonist of its receptor during the secondary response to CPA. This suggests a direct and dynamic interaction between one or more of the components of the two receptor coupling systems. Fifthly, this interaction did not involve a simple switch in the coupling of the PT-sensitive G-protein away from adenylate cyclase to PLC. This implies that an additional coupling mechanism is brought into play in the presence of the primary agonist. The mechanism underlying this interaction is unclear. However, it is noteworthy that exogenous $\beta\gamma$ subunits potentiated an agonist/GTP-dependent activation of PLC when added to turkey erythrocyte membrane preparations [44]. This was manifest as an increased capacity, rather than a higher affinity, of the occupied receptor for PLC. These findings have recently been extended with the demonstration that some isoforms of PLC may be activated directly by $\beta\gamma$ subunits *in vitro* [45]. It is therefore possible that $\beta\gamma$ subunits released from G-proteins coupled to the A1 receptor might play a role in potentiating the response to carbachol. This role is likely to be more complex than suggested by the results found *in vitro*, and further work will be required to establish the applicability of these findings to the responses described in the present study.

It might have been predicted that the demonstrated cross-talk between muscarinic and adenosine A1 receptors would have

resulted in a corresponding potentiation of insulin release. On the other hand, given the potent inhibitory effects of CPA on insulin secretion evoked by nutrient stimuli, an inhibition of the secretory response to carbachol might equally have been expected. In fact no significant alteration was observed at all (Figure 9b). The simplest explanation for this apparent anomaly is that, whereas with nutrient secretagogues CPA exerts two inhibitory effects (on Ca^{2+} influx and the distal step), with carbachol or vasopressin the distal response is counteracted by potentiation of PtdInsP_2 hydrolysis. However, direct evidence for this hypothesis is lacking, since we were unable to dissociate these positive and negative inputs using, for example, PT pretreatment (results not shown). It is possible that the differential sensitivity of nutrients and PLC-linked agonists to inhibition might be of physiological relevance. Indeed, these results might imply that the cephalic phase of insulin secretion represents less a priming of the β -cell to a following nutrient stimulus, than the removal of a tonic inhibitory effect due to the local mediator adenosine. Such a prediction will need to be tested in whole animals. It is also noteworthy that the present study could explain earlier findings showing that adrenaline was much more effective in inhibiting the secretory response to glucose than to acetylcholine in perfused rat islets [46]. This implies that our results with A1-agonists might equally apply to other agents which act through receptors linked to inhibitory G-proteins. In support of this possibility is the brief comment made in a recent study that $1\ \mu\text{M}$ adrenaline, if added to the hamster β -cell line HIT after carbachol, slightly enhanced $[\text{Ca}^{2+}]_i$ [19].

In conclusion, the results presented here demonstrate that A1-adenosine agonists not only inhibit Ca^{2+} influx but also promote its mobilization from intracellular stores, provided a primary agonist linked to PtdInsP_2 hydrolysis is also present. This requires a dynamic interaction between the components of the two signalling systems. As a consequence of the described responses, occupation of A1 receptors will lead to an inhibition of nutrient-stimulated insulin release, but not the secretory response due to agonists which act via the hydrolysis of PtdInsP_2 .

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