Role of Ins(1,4,5) P_3 , cADP-ribose and nicotinic acid–adenine dinucleotide phosphate in Ca^{2+} signalling in mouse submandibular acinar cells

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cADP-ribose (cADPr) and nicotinic acid–adenine dinucleotide phosphate (NAADP) are two putative second messengers; they were first shown to stimulate Ca^{2+} mobilization in sea urchin eggs. We have used the patch-clamp whole-cell technique to determine the role of cADPr and NAADP in relation to that of Ins(1,4,5) P_3 in mouse submandibular acinar cells by measuring agonist-evoked and second-messenger-evoked changes in Ca^{2+} -dependent K^+ and Cl^- currents. Both Ins(1,4,5) P_3 and cADPr were capable of reproducing the full range of responses normally seen in response to stimulation with acetylcholine (ACh). Low concentrations of agonist (10–20 nM ACh) or second messenger [1–10 μ M Ins(1,4,5) P_3 or cADPr] elicited a sporadic transient activation of the Ca^{2+} -dependent currents; mid-range concentrations [50–500 nM ACh, 50 μ M Ins(1,4,5) P_3 or 50–100 μ M cADPr] elicited high-frequency (approx. 2 Hz) trains of current spikes; and high concentrations [more than 500 nM ACh, more

than 50 μ M Ins(1,4,5) P_3 or more than 100 μ M cADPr] gave rise to a sustained current response. The response to ACh was inhibited by antagonists of both the Ins(1,4,5) P_3 receptor [Ins(1,4,5) P_3 R] and the ryanodine receptor (RyR) but could be completely blocked only by an Ins(1,4,5) P_3 R antagonist (heparin). NAADP (50 nM to 100 μ M) did not itself activate the Ca^{2+} -dependent ion currents, nor did it inhibit the activation of these currents by ACh. These results show that, in these cells, both Ins(1,4,5) P_3 R and RyR are involved in the propagation of the Ca^{2+} signal stimulated by ACh and that cADPr can function as an endogenous regulator of RyR. Furthermore, although NAADP might have a role in hormone-stimulated secretion in pancreatic acinar cells, it does not contribute to ACh-evoked secretion in submandibular acinar cells.

Key words: Cl^- current, exocrine, K^+ current, patch-clamp.

INTRODUCTION

The secretion of fluids and electrolytes in salivary gland acinar cells is driven by the activation of Ca^{2+} -dependent K^+ and Cl^- channels [1,2]. The penultimate step in stimulus–secretion coupling is an increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$), the initial phase of which stems from release from intracellular Ca^{2+} stores, probably the endoplasmic reticulum [3]. The Ca^{2+} signal might also be supplemented by Ca^{2+} influx via a store-operated Ca^{2+} influx pathway [4,5]. There is a growing body of evidence that pulsatile, localized Ca^{2+} release from intracellular stores is an integral and necessary part of the signal transduction pathway in many cell types, including polarized acinar cells [6–8].

Positive feedback is a characteristic feature of intracellular signalling events mediated by changes in $[Ca^{2+}]_i$. Both localized ‘spikes’ and cell-wide increases in $[Ca^{2+}]_i$ depend on Ca^{2+} -induced Ca^{2+} release (CICR), the process whereby elemental Ca^{2+} release events trigger further Ca^{2+} release from intracellular stores to generate a functional Ca^{2+} signal. Current models of the Ca^{2+} signalling process postulate that the probability of an elemental Ca^{2+} release event triggering CICR depends on the level of the intracellular agonist responsible for gating the Ca^{2+} channel [6]. In principle, the Ins(1,4,5) P_3 receptor [Ins(1,4,5) P_3 R] has all the properties necessary to support CICR-generated Ca^{2+} signals by itself; all subtypes of Ins(1,4,5) P_3 R are sensitive to increased $[Ca^{2+}]_i$ [9–11] and Ins(1,4,5) P_3 levels are directly controlled by the binding of an extracellular agonist to a receptor. Nevertheless, there is growing evidence of multiple mechanisms for both the initiation and propagation of CICR.

Recent studies in pancreatic acinar cells have proposed roles for the putative second messengers, cADP-ribose (cADPr)

[12–15] and nicotinic acid–adenine dinucleotide phosphate (NAADP) [16–19] in addition to Ins(1,4,5) P_3 [20] in the oscillatory signal transduction process triggered by cholecystokinin (CCK).

There are many morphological and functional similarities between submandibular and pancreatic acinar cells; furthermore, both cell types respond to stimulation by acetylcholine (ACh) with trains of short transient increases in $[Ca^{2+}]_i$ that can be seen to originate at the apical pole of the cell [8,21] (A. R. Harmer, unpublished work) and that can be monitored by measuring Ca^{2+} -dependent ion currents via the patch-clamp whole-cell technique [22,23]. However, mouse submandibular acinar cells do not respond to stimulation with CCK, nor do they exhibit the sinusoidal oscillatory component of the $[Ca^{2+}]_i$ and Ca^{2+} -dependent current response characteristic of CCK stimulation in mouse pancreatic acinar cells.

Mouse submandibular acinar cells are therefore a well-characterized but less complex system than pancreatic acinar cells in which to determine whether cADPr and NAADP are involved in neurally controlled, ACh-mediated signal transduction or whether one or both of these second messengers have a role only in hormone-mediated secretion. To make this determination, we applied the patch-clamp whole-cell technique to acutely isolated mouse submandibular cells to compare the increase in Ca^{2+} -dependent ion channel activity stimulated by infusion of second messenger into the cell to that stimulated by ACh.

MATERIALS AND METHODS

Adult male CD1 mice were killed by cervical dislocation; pancreatic and submandibular cells were isolated by digestion

Abbreviations used: ACh, acetylcholine; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; cADPr, cADP-ribose; CCK, cholecystokinin; CICR, Ca^{2+} -induced Ca^{2+} release; Ins(1,4,5) P_3 R, Ins(1,4,5) P_3 receptor; NAADP, nicotinic acid–adenine dinucleotide phosphate; RyR, ryanodine receptor.

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with collagenase (Worthington Biochemical Corporation, Lakewood, NJ, U.S.A.) in extracellular medium containing 1 mM Ca^{2+} , as described previously [18,23]. For submandibular cells this was followed by a short treatment (0.5–2 min) with 0.8 mg/ml trypsin. After dispersal, cells were suspended in serum-free Dulbecco's modified Eagle's medium/F12 medium (1:1, v/v) and were used within 3 h.

For each experiment, cells were allowed to settle on a coverslip coated with poly-(L-lysine) (Sigma, Poole, Dorset, U.K.) that formed the base of a perfusion chamber and was placed on the stage of an inverted microscope (CM; Olympus Optical Company, Southall, Middx., U.K.). Cells were superfused continuously at 0.5 ml/min from one of several parallel superfusion pipettes. The solution bathing the cell could be changed in 1–2 s. All experiments were performed at $24 \pm 2^\circ\text{C}$.

The patch-clamp whole-cell configuration was achieved with single cells by using 2–4 M Ω patch-clamp pipettes pulled from borosilicate glass capillaries (Clark Electromedical Instruments, Reading, Berks., U.K.). Access resistance through the patch pipette was approx. 3-fold that of the pipette itself. Cells were voltage clamped to -40 mV with an Axopatch 200a patch-clamp amplifier (Axon Instruments, Foster City, CA, U.S.A.). K^+ and Cl^- currents were measured separately by pulsing to 0 and -80 mV respectively for 100 ms, twice every 1 s. Currents were digitized with a CED 1401 interface (Cambridge Electronics Design, Cambridge, U.K.) and stored and analysed on a PC with custom written software [24].

The patch-clamp pipette contained (in mM): 140 KCl, 1.13 MgCl_2 , 10 glucose, 0.5 EGTA and 1 ATP, buffered to pH 7.4 with 10 mM Hepes. Where indicated in the text and Figure legends the pipette also contained various concentrations of $\text{Ins}(1,4,5)\text{P}_3$, heparin (Na^+ salt, low Ca^{2+}), cADPr, 8-amino-cADPr and NAADP. In experiments performed with cADPr, 8-amino-cADPr and NAADP, glucose was omitted from the patch pipette solution to avoid any possible inhibitory effects [14]. cADPr, NAADP and heparin were obtained from Sigma, 8-amino-cADPr was from Molecular Probes and $\text{Ins}(1,4,5)\text{P}_3$ was a gift from R. F. Irvine (Cambridge, U.K.).

The extracellular bathing solution contained (in mM): 140 NaCl, 4.7 KCl, 1.13 MgCl_2 , 1.0 CaCl_2 and 10 glucose, buffered to pH 7.4 with 10 mM Hepes.

These experiments took advantage of the access to the cell interior offered by the patch-clamp whole-cell technique to infuse substances into the cell. In all such experiments these substances were added to the intracellular medium at the start of the experiment and thus entered the cell as soon as the breakthrough to the whole-cell condition was achieved.

RESULTS

We have previously reported repetitive transient activation of the Ca^{2+} -dependent K^+ and Cl^- currents elicited with 50 nM ACh [23]. The data in Figure 1 are the first report of the concentration–response relationship to low concentrations of ACh in these cells. There is evidence of a concentration-dependent frequency response only at the extreme ends of the concentration range, where a very low dose of ACh (10–20 nM) generated only sporadic current transients and high (micromolar) ACh concentrations elicited a sustained (fused) activation of the currents. Within the extremes, as shown in Figures 1(B) and 1(C), the frequency of the current transients was largely independent of concentration. However, there was a consistent change in the distribution of K^+ and Cl^- channel activation as ACh concentration was increased. At low ACh doses (10–20 nM), only the Cl^- current was activated (Figure 1A); at intermediate ACh

Figure 1 ACh concentration–response

K^+ (upper trace) and Cl^- (lower trace) currents measured in single mouse submandibular cells in response to stimulation by 20 nM (A), 50 nM (B), 500 nM (C), 1 μM (D) and 5 μM (E) ACh. The broken line indicates zero current. The data shown are typical of 60 similar experiments. The scale in (E) applies to all panels.

concentrations (20–50 nM), repetitive transient activation of both the K^+ and Cl^- currents occurred (Figure 1B); at 500 nM ACh, sustained activation of the K^+ current was observed in conjunction with transient activation of the Cl^- current (Figure 1C); and sustained activation of both currents was generated by a micromolar dose (Figure 1D).

The binding of ACh to the muscarinic M_3 receptor is known to activate phospholipase C via a G-protein and to increase $\text{Ins}(1,4,5)\text{P}_3$ levels in the cell [6]. The receptor activation step of the signal transduction process can be circumvented by infusing $\text{Ins}(1,4,5)\text{P}_3$ directly into the cell via the patch pipette [20]. Figure 2 shows the dose-dependent pattern of activation of both Ca^{2+} -dependent currents after the infusion of $\text{Ins}(1,4,5)\text{P}_3$. $\text{Ins}(1,4,5)\text{P}_3$

Figure 2 Ins(1,4,5) P_3 concentration–response

K^+ (upper trace) and Cl^- (lower trace) currents measured in single mouse submandibular cells in response to intracellular perfusion with 0 (A), 1 (B), 10 (C), 50 (D) and 100 (E) μM Ins(1,4,5) P_3 . The dotted line indicates zero current. Of 29 cells, 18 responded to 1 μM Ins(1,4,5) P_3 ; 17 of 27 cells responded to 10 μM Ins(1,4,5) P_3 ; 7 of 7 cells responded to 50 μM Ins(1,4,5) P_3 ; and 6 of 6 cells responded to 100 μM Ins(1,4,5) P_3 . The data shown are typical of those cells that responded at each concentration.

was effective over two orders of magnitude from 1 to 100 μM . The pattern of response to Ins(1,4,5) P_3 was very similar to that seen in response to ACh in both the limited frequency response (compare Figures 2B and 2C) and the distribution of K^+ and Cl^- current activation as concentration was increased. Low concentrations of Ins(1,4,5) P_3 (1–10 μM) caused preferential activation of the Cl^- current, while a higher concentration (50–100 μM) activated both currents (compare Figures 2B and 2C with Figures 2D and 2E).

cADP-ribose has been shown to increase the sensitivity of the ryanodine receptor (RyR) to Ca^{2+} and thus to promote CICR. It

Figure 3 cADPr concentration–response

K^+ (upper trace) and Cl^- (lower trace) currents measured in single mouse submandibular cells in response to intracellular perfusion with 1 (A), 10 (B), 50 (C), 100 (D) and 500 (E) μM cADPr. The dotted line indicates zero current. Of 25 cells, 10 responded to 1 μM cADPr; 17 of 27 cells responded to 10 μM cADPr; 9 of 19 cells responded to 50 μM cADPr; 7 of 20 cells responded to 100 μM cADPr; and 8 of 20 cells responded to 500 μM cADPr. The data shown are typical of those cells that responded at each concentration.

is thought to act as the endogenous regulator for the RyR [25]. The data in Figure 3 result from intracellular perfusion of the cells with cADPr and they provide evidence that it is capable of acting as an endogenous regulator and of mobilizing intracellular Ca^{2+} to activate the Ca^{2+} -dependent currents in submandibular acinar cells. The response to increased concentrations of cADPr was similar to the response to ACh and Ins(1,4,5) P_3 in terms of both frequency and distribution of the K^+ and Cl^- currents. Furthermore, the presence of a low concentration of cADPr (10 μM) greatly amplified the response to a threshold dose of ACh (Figure 4, compare with Figure 1A).

The infusion of 10–500 μM NAADP, which has been shown to mobilize Ca^{2+} and to cause the repetitive transient activation of Ca^{2+} -dependent currents in mouse pancreatic acinar cells [18,19]

Figure 4 Response to ACh in the presence of cADPr

K⁺ (upper trace) and Cl[−] (lower trace) currents measured in a single mouse submandibular cell in response to intracellular perfusion with 10 μ M cADPr and subsequent application of 10 nM ACh. The dotted line indicates zero current.

Figure 5 NAADP concentration–response

(A) Cl[−] current measured in single pancreatic acinar cells in two separate experiments in response to intracellular perfusion of 50 nM NAADP. (B–F) K⁺ (upper trace) and Cl[−] (lower trace) currents measured in single mouse submandibular cells in response to intracellular perfusion with 50 nM (B), 75 nM (C), 500 nM (D), 1 μ M (E) and 100 μ M (F) NAADP. The broken line indicates zero current. The data shown are typical of 68 similar experiments.

(Figure 5A), did not cause activation of the K⁺ and Cl[−] currents in submandibular acinar cells. In the presence of NAADP at concentrations less than 1 μ M, very infrequent transient activation of the Cl[−] current but not the K⁺ current was observed

in 40% of 68 cells, as shown in Figures 5(B)–5(D). Mouse submandibular acinar cells can generate occasional, isolated Cl[−] current ‘spikes’ in the absence of stimulation (P. M. Smith, unpublished work); the Cl[−] current ‘spikes’ in Figure 5 were indistinguishable from these ‘spontaneous’ events. No current activation was observed after the infusion of 1 or 100 μ M NAADP (Figures 5E and 5F).

NAADP has been shown to have a novel self-inactivating mechanism in the pancreas that results in the inhibition of the CCK-evoked Ca²⁺ signal at high NAADP concentrations [18]. The data in Figure 6(A) show that, even at a concentration of 100 μ M, NAADP did not inhibit repetitive transient activation of the K⁺ and Cl[−] currents stimulated by 20 nM ACh. Inhibition of the ACh-evoked response could be achieved by infusion of the Ins(1,4,5) P_3 receptor antagonist heparin, as shown in Figure 6(B). The action of the cADPr receptor inhibitor 8-amino-cADPr was less pronounced. The data in Figure 6(D) show that stimulation with ACh could evoke transient activation of the K⁺ and Cl[−] currents in the presence of 18 μ M 8-amino-cADPr; however, when these data are compared with those in Figure 6(C), which were obtained immediately after those in Figure 5(D), it is clear that the response to ACh has been attenuated by the cADPr receptor antagonist. Similar attenuation of the ACh response was seen in 64% of 28 experiments. Despite consistently inhibiting the response to even a high dose of ACh (500 nM), infusion of 8-amino-cADPr (18 μ M) failed to abolish completely the response to even a threshold concentration of ACh (10–20 nM).

DISCUSSION

Alternate CICR pathways were first described as ‘redundant’ [26], implying that one or other of the pathways alone could accommodate the normal process of signal transduction. A superficial analysis of the results that we present here falls within this category. We have shown previously that submandibular acinar cells respond to a low dose of agonist with repetitive transient ‘spikes’ in the Ca²⁺-dependent K⁺ and Cl[−] currents [23,27]. Here we show that this pattern of response could be elicited by the activation of either Ins(1,4,5) P_3 R or RyRs by the direct infusion of Ins(1,4,5) P_3 or cADPr respectively. Furthermore, we show that either second messenger was capable of mimicking the complete dose response elicited by ACh, from sparse transient activation to a sustained elevation of the currents.

A greater insight into the roles of Ins(1,4,5) P_3 and cADPr in control of the intracellular signal can be obtained from an analysis of the action of the inhibitors of these second messengers. Both heparin, which inhibits Ins(1,4,5) P_3 binding [28], and 8-amino-cADPr, which competes with cADPr for the RyR [29], inhibited the activation of Ca²⁺-dependent currents by ACh. The striking difference between these two inhibitors was that, whereas 8-amino-cADPr consistently decreased the response to ACh, heparin was capable of abolishing it completely. This observation implies that, whereas both Ins(1,4,5) P_3 R and RyR are involved in the propagation of the ACh-evoked Ca²⁺ signal in these cells, there is an obligate role for Ins(1,4,5) P_3 -mediated Ca²⁺ release in initiating the response. This conclusion is consistent with the observations that RyRs are mainly located at the basal pole of acinar cells [30,31] and that Ca²⁺ signals originate at the apical pole of the cell [21] (A. R. Harmer, unpublished work).

Although these results might suggest a subordinate or ‘redundant’ role for cADPr and the RyR, there are other more interesting possibilities. Our results show that the level of cADPr within the cell can determine the magnitude of the response to a given concentration of ACh. The key to understanding what

Figure 6 Effect of inhibitors of Ins(1,4,5) P_3 , cADPr and NAADP

K⁺ (upper trace) and Cl[−] (lower trace) currents measured in single mouse submandibular cells showing the response to 20 nM ACh in the presence of 100 μ M NAADP (**A**), the response to 20, 50 and 500 nM ACh in the presence of 400 μ g/ml heparin (**B**), the response to 20, 50 and 500 nM ACh (**C**) and the response to 20, 50 and 500 nM ACh in the presence of 18 μ M 8-amino-cADPr (**D**). The data for (**C**) and (**D**) were obtained immediately after one another from the same batch of cells. A similar attenuation was observed in 68% of 28 experiments. The broken line indicates zero current.

physiological relevance this might have lies with the processes that normally regulate cellular levels of cADPr. cADPr is synthesized from NAD by ADP ribosyl cyclase [25,32] under the control of cGMP [25,33]. One factor controlling cGMP levels in acinar cells is nitric oxide (NO) [34–37]. NO levels are in turn dependent on [Ca²⁺]_i [34,37], which is regulated by Ins(1,4,5) P_3 and indeed cADPr. What saves this process from being simply a long-winded positive feedback loop is the ability of NO to cross cell membranes; thus the activity in one cell has the capacity to influence that of another. There is evidence that the inhibition of NO synthase can inhibit salivation in rats [38]. Our results demonstrate that this inhibition could be accomplished by the down-regulation of cADPr production.

One physiological role for communication between acinar cells, mediated by NO and cADPr, might be to co-ordinate activity between different cells within an acinus. Alternatively,

this pathway could permit the co-ordination of activity between duct and acinar cells. This latter possibility is supported by measurements of NO synthase activity made in salivary glands, which showed the highest activity of this enzyme in duct cells [38].

NAADP, like cADPr, is produced by ADP ribosyl cyclase [39]. NAADP has also been proposed as a Ca²⁺-mobilizing second messenger [18] but not one that directly effects Ca²⁺ release from stores gated by Ins(1,4,5) P_3 R or RyR; rather, NAADP is thought to stimulate Ca²⁺ release from a separate pool of stored Ca²⁺ [17,40]. Nevertheless, NAADP could easily have a role in intracellular Ca²⁺ signalling through the mechanism of CICR. Propagation and amplification of the Ca²⁺ signal by both Ins(1,4,5) P_3 R and RyR could occur after an initiating event triggered by NAADP. Such a hypothesis has been proposed to account for the Ca²⁺ signal and the consequent activation of

Ca²⁺-dependent ion channels seen in pancreatic acinar cells stimulated with CCK [18,19]. However, our results show that it is not possible to stimulate Ca²⁺-dependent ion channel activity by the intracellular infusion of NAADP over a wide range of concentrations; we were also unable to show any inhibitory action of the high concentration of NAADP (100 µM) shown previously to inhibit the response to CCK in pancreatic acinar cells [19].

NAADP might have a role in hormone-mediated Ca²⁺ signalling in pancreatic acinar cells; however, it is not involved in the neurally activated signal transduction process in submandibular acinar cells. Furthermore, it might be the contribution of NAADP-gated stores that permits different patterns of Ca²⁺ signal in pancreatic acinar cells but not in submandibular acinar cells.

Our results support our previous hypothesis that both Ins(1,4,5)P₃R and RyR have a role in Ca²⁺-mediated signal transduction in mouse submandibular acinar cells. We have now demonstrated that cADPr can function as an endogenous agonist for the RyR in these cells and that activation of the RyR by cADPr can act synergistically with ACh-mediated activation of Ins(1,4,5)P₃R. Our results do not support a role for NAADP-mediated Ca²⁺ release in the signal transduction process in these cells.

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