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ACETYLCHOLINE- AND CAFFEINE-EVOKED REPETITIVE TRANSIENT Ca^{2+}-ACTIVATED K^+ AND Cl^- CURRENTS IN MOUSE SUBMANDIBULAR CELLS

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SUMMARY

1. Resting and acetylcholine-induced membrane currents were measured in single mouse submandibular acinar cells using the patch-clamp whole-cell current recording technique.

2. Micromolar ACh activated a large, sustained outward, Ca^{2+}-dependent K^+ current and a single transient inward Ca^{2+}-dependent Cl^-current.

3. Nanomolar ACh induced a series of transients in both the K^+ and Cl^- currents; Cl^- current activation was now observed throughout the period of agonist application. We consider this repetitive transient current activation better able to support sustained fluid and electrolyte secretion than the response elicited by a high dose of agonist.

4. Repetitive K^+ and Cl^- current transients were also induced by 1 mM-caffeine, consistent with caffeine-induced Ca^{2+} release from the Ca^{2+}-sensitive Ca^{2+} stores which are thought to comprise part of the pathway for activation of secretion.

5. The ACh-induced current transients were inhibited by 10 mM-caffeine, 100 μM-IBMX and 10 μM membrane-permeable cyclic AMP. Therefore, it seems likely that caffeine is able to inhibit agonist-induced calcium mobilization via a cyclic AMP-dependent pathway.

INTRODUCTION

In salivary and lacrimal acinar cells a variety of neurotransmitters and hormones act as secretagogues by activation of a common transduction mechanism. The receptors for these agonists are coupled, via G proteins, to regulate the activity of the enzyme phospholipase C (PLC). Receptor occupation stimulates enzyme activity and the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) is hydrolysed to yield the two second messengers inositol 1,4,5-trisphosphate (InsP_3) and diacylglycerol (Berridge, 1987; Flemming, Sliwinski-Lis & Burke, 1989). InsP_3 (Berridge, 1987; Petersen & Wakui, 1990) and possibly its metabolites (Morris, Gallacher, Irvine & Petersen, 1987), act to release Ca^{2+} from intracellular stores and ultimately to promote Ca^{2+} entry across the cell surface membrane.

In recent years, two techniques have been used in exocrine cells to monitor changes

in $[Ca^{2+}]_i$ in response to phosphoinositide-coupled agonists. These are: direct measurement using fluorescent indicators for Ca^{2+} (Gray, 1988; Yule & Gallacher, 1988) and indirect measurement using patch-clamp whole-cell recording of Ca^{2+}-activated conductances (Morris *et al.* 1987; Wakui, Potter & Petersen, 1989). Both techniques have revealed that the increase in Ca^{2+} and activation of Ca^{2+}-dependent conductances can be of an oscillatory nature. These oscillatory responses are currently the subject of intense investigation. One hypothesis to account for oscillatory increases in $[Ca^{2+}]_i$ involves two separate Ca^{2+} pools (Kuba, 1981; Goldbeter, Dupont & Berridge, 1990; Petersen & Wakui, 1990), in which InsP_3 causes Ca^{2+} release from the first pool and this Ca^{2+} release triggers further Ca^{2+} release from a Ca^{2+}-dependent Ca^{2+} pool, analogous to that described in muscle sarcoplasmic reticulum (Ford & Podolsky, 1970). It is possible to model interaction between these two pools in such a fashion as to account for oscillatory increases in $[Ca^{2+}]_i$ stimulated by a constant level of InsP_3 in accordance with experimental results showing repetitive Ca^{2+} spikes during continuous intracellular infusion of InsP_3 (Wakui *et al.* 1989).

The final step of stimulus–secretion coupling is initiation of the secretory process itself. Activation of an apical membrane anion conductance is thought to be the proximate cause of fluid and electrolyte secretion in many secretory epithelia (Welsh, 1987; Petersen & Gallacher, 1988). In exocrine glands Maruyama, Gallacher & Petersen (1983) showed that activation of basolateral membrane K^+ channels was an integral part of the secretory process. Thus, in acinar cells, activation of the K^+ channels maintains a favourable electrochemical gradient for Cl^- efflux via Cl^- channels in the luminal membrane. As K^+ is recycled across the basolateral membrane by the Na^+–K^+ ATPase and the Na^+–K^+–Cl^- co-transporter, the only net transcellular transport is Cl^-, from interstitium to lumen. The resultant luminal negativity draws Na^+ into the lumen via the paracellular pathway and creates the osmotic conditions necessary for fluid secretion. Activation of the K^+ and Cl^- conductances are therefore key elements in the initiation and maintenance of the secretory process.

Previously, in lacrimal and salivary glands, the effects of micromolar concentrations of cholinergic agonists have been studied (Nishiyama, & Petersen, 1974; Putney, 1977; Iwatsuki, Maruyama, Matsumoto & Nishiyama, 1985; Morris *et al.* 1987; Sasaki & Gallacher, 1990). A common factor in these studies is a sustained increase in K^+ conductance. However, when the agonist-induced increase in Cl^- conductance was measured it was found to be transient and rapidly returned to pre-stimulus levels. This does not meet the criteria for secretion outlined above.

We have investigated the effects of lower, nanomolar concentrations of ACh. At these low concentrations ACh again evokes both outward and inward current responses; the responses are, however, composed of a series of brief transient increases in conductance and, most importantly, the Cl^- current transients are sustained throughout the period of agonist application. Pulsatile Ca^{2+} signalling in submandibular cells may provide a mechanism for extending the period of activation of the apical membrane anion conductance beyond the 1 or 2 min observed with high-dose agonist stimulation.

We also report that caffeine can both stimulate current responses identical to those

of ACh and at higher concentrations inhibit the ACh-induced responses. The stimulatory response is consistent with caffeine-dependent release of Ca^{2+} from a Ca^{2+}-dependent Ca^{2+} pool and we provide evidence that at least one pathway for the inhibitory response is via phosphodiesterase inhibition and stimulation of cyclic AMP-dependent kinases.

METHODS

Cell preparation

Adult male mice were killed by stunning and cervical dislocation. Submandibular glands were excised and cut into small pieces. Isolated acini were prepared by collagenase (Worthington Diagnostic USA, 200 units/ml) digestion in a shaking water bath at 37 °C for 20 min. The tissue suspension was vigorously shaken at 5 min intervals during the 20 min incubation. Following digestion, the cells were spun down (1000 r.p.m. for 1 min) and washed twice. The resulting cell suspension was incubated in 10 ml of physiological saline for up to 2 h before use. For the experiments a 0·5 ml aliquot was transferred to a small plastic Petri dish on the stage of an inverted microscope. The cells were allowed to settle and then washed twice with 3 ml of saline. Single acinar cells, viewed at ×400 magnification, were selected for experimental purposes. The 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 carried out at 24 ± 2 °C.

Electrical recordings

Whole-cell currents were measured using fire-polished patch-clamp pipettes pulled from Assistent micro haematocrit tubing using a David Kopf Instruments vertical pipette puller to an electrical resistance of 2–5 MΩ when filled with a 140 mM-KCl solution.

Measurements were made via an LM-EPC7 (List Electronics, Darmstadt, FRG) patch-clamp amplifier, signals were digitized by a Cambridge Electronic Design 1401 interface and recorded on hard disc (Opus PC VI, IBM AT compatible microcomputer). The data were also simultaneously recorded, with much greater time resolution, on a Digital Audio Tape (DAT) recorder (Biologic). Software for pulse generation and data collection was written for this application by P. M. Smith.

Protocols in which currents were measured 'simultaneously' at three different voltages by using short pulses to change the holding potential have been previously described (Findlay, 1984). These experiments differ from those previously published in the means by which the data were collected.

Briefly, the software replaced the signal generator and the chart recorder used in conventional patch-clamp rigs. For whole-cell, pulse-protocol experiments, the oscilloscope and the tape-recorder were also largely redundant.

In the protocols used in these experiments, cells were voltage clamped to a holding potential of −40 mV by the List EPC7 and two 100 ms long 40 mV hyperpolarizing and depolarizing voltage pulses were applied every 500 ms. The resultant currents were collected continuously at 4 kHz and displayed on the computer monitor. Those data collected in a 50 ms period in the middle of each voltage pulse were averaged to give the mean current at that voltage (thus avoiding on/off transients). Together with the mean resting current, averaged over a 50 ms period after the pulses, these average values were saved twice a second. Thus 4000 values/s of raw data were compressed on-line to 6 values/s without losing any useful information.

Alternatively, *all* the data could be saved by sampling continuously at 2 kHz. As this requires 250 K of disc space per minute, the DAT recorder provided the most economical way of storing all the data in real time; important records could be replayed from tape and then digitized.

Solutions

Physiological saline contained (in mM): 140 NaCl, 4·7 KCl, 1·13 $MgCl_2$, 1·2 $CaCl_2$, 10 glucose, buffered to pH 7·2 with 10 mM-HEPES.

The standard internal (pipette) solution contained (in mM): 140 KCl, 1·13 $MgCl_2$, 10 glucose, 0·5 EGTA, 1·0 ATP, buffered to pH 7·2 with 10 mM-HEPES.

Low-Cl^- solutions were made by replacement of NaCl or KCl with sodium or potassium gluconate respectively.

Osmolarity was adjusted to 290±5 mosM for all solutions. All chemicals and reagents were of Analar grade and obtained from BDH. Drugs were obtained from Sigma Chemical Company UK.

RESULTS

ACH-induced K^+ and Cl^- currents

It has been demonstrated previously that submandibular cells have both the voltage- and Ca^{2+}-activated (maxi) K^+ channels (Maruyama *et al.* 1983; Gallacher & Morris, 1986), and a Ca^{2+}-activated Cl^- channel (Cook, Day, Champion & Young, 1988). The hyperpolarizing and depolarizing pulse protocol used in these experiments was intended to show changes in both K^+ and Cl^- conductance independently of one another. Thus K^+ currents were measured at 0 mV, the reversal potential for symmetrical Cl^- solutions, and Cl^- currents at −80 mV, the reversal potential for K^+. Figure 1 shows the response of mouse submandibular cells to a 'high' dose (500 nM–1 μM) of ACh. Like most previous studies (Nishiyama & Petersen, 1974; Putney, 1977; Iwatsuki *et al.* 1985; Morris *et al.* 1987; Sasaki & Gallacher, 1990) a 'high' dose of ACh caused a large initial transient response which declined to a lower sustained level over several minutes; removal of ACh caused an immediate return to pre-stimulus levels (number of experiments (n) = 15). Figure 1*B* shows confirmation that the ACh-induced currents measured at 0 and −80 mV represent movement of only K^+ and Cl^- ions respectively. As the resting Cl^- conductance was very low, there was very little inward current measured at −80 mV in unstimulated cells, and replacement of Cl^- in both pipette and bathing solutions with gluconate had no obvious effect. However, removal of Cl^- abolished the ACh-induced inward current (n = 5). The K^+ channel blocker tetraethylammonium (TEA) abolished both the resting K^+ conductance and prevented any ACh stimulation of outward current. In the presence of TEA and in the absence of Cl^-, ACh had no effect on either outward or inward currents (n = 2). Figure 1*C* demonstrates the Ca^{2+} dependence of these currents; when the EGTA concentration in the pipette was increased from 0·5 to 2·5 mM, ACh did not alter either the K^+ or Cl^- currents (n = 3). Therefore, the response to ACh seen under normal ionic conditions consists only of Ca^{2+} activated K^+ and Cl^- currents.

ACh-induced transients K^+ and Cl^- currents

A low (10–50 nM) dose of ACh gave rise to irregular, repetitive, transient activation of the K^+ and Cl^- currents (n > 50). In some cells, a very low (< 20 nM) dose of ACh caused small infrequent (one every 2–5 s) current transients and a return to baseline values between transients. When this type of response was observed, raising the dose of ACh (20–50 nM) increased both the size and frequency of the current transients. Figure 2*A* shows the most typical response where the frequency of the transients was too fast to allow a return to baseline values and therefore these transients appear superimposed on top of a sustained increase in current. There was usually more of a sustained component to the K^+ current response than to the Cl^- current response but the transient component of the Cl^- currents was usually the larger of the two. In fact, the peak values of the transient Cl^- currents elicited by nanomolar concentrations of ACh were often much larger than the peak Cl^- currents produced in response to micromolar ACh (Figs 1 and 2). The type of response shown in Fig. 2 showed little

obvious concentration dependence in either magnitude or frequency until the ACh dose was increased past 500 nM and the response was transformed into the 'sustained' type shown in Fig. 1.

In some cells, following the first exposure to ACh, there was a lag of up to 30 s in the onset of the response. The second and subsequent responses to the same

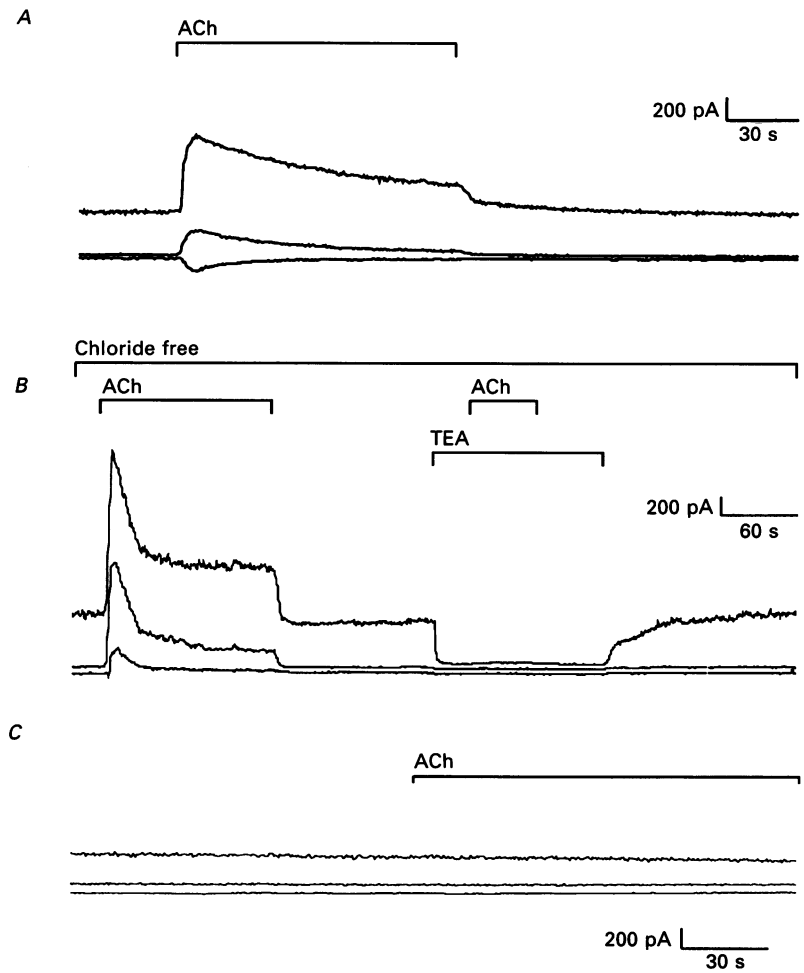

Fig. 1. *A*, transient and sustained current responses measured at 0 mV (top line, outward current), −40 mV (middle line, zero current) and −80 mV (bottom line, inward current) in response to 500 nM-ACh stimulation. *B*, currents measured at 0 mV (top line), −40 mV (middle line) and −80 mV (bottom line) when gluconate replaced Cl^- as the major anion in both the bath and pipette solutions. ACh (50 nM) produced both transient and sustained outward currents, but no inward currents, even at −80 mV. TEA (5 mM) abolished both the resting and ACh-stimulated outward currents. *C*, currents measured at 0 mV (top line), −40 mV (middle line) and 80 mV (bottom line) when EGTA in the pipette was raised from 0·5 to 2·5 mM. No effect of 50 nM-ACh was observed at any voltage.

concentration of ACh occurred with a much reduced latency. These responses were qualitatively similar to the initial response although sometimes smaller; this was most evident with concentrations of ACh of 50 nM or more. The ACh response could be maintained for up to 10–15 min or until ACh was removed. Removal of ACh

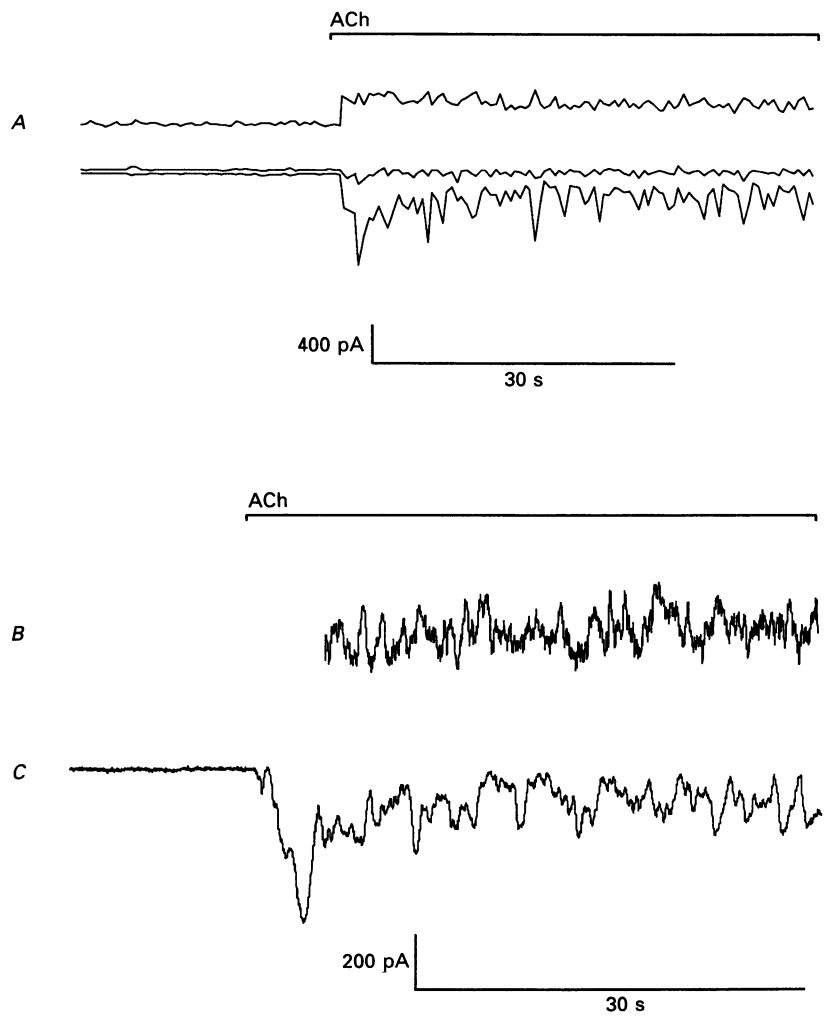

Fig. 2. *A*, pulsatile currents measured at 0 mV (top line), −40 mV (middle line) and −80 mV (bottom line) in response to 50 nM-ACh. *B*, pulsatile currents following 50 nM-ACh stimulation measured without using pulse protocol, at a holding potential of 0 mV. *C*, pulsatile currents following 50 nM-ACh stimulation measured without using pulse protocol, at a holding potential of −80 mV. Unlike the pulse-protocol figures, *B* and *C* are not synchronous. *B* and *C* represent portions of a continuous trace sampled at 2 kHz. Both traces are from the same cell; *B* was obtained immediately after *C*.

always caused a return of both K^+ and Cl^- currents to baseline values. The return to baseline values occurred over a few seconds, consistent with the time required to wash away the ACh. The repetitive transient response to low concentrations of ACh

was not acutely dependent on extracellular Ca^{2+} as transients could be observed in the continued presence of ACh 10 min after the Ca^{2+} in the external bathing solution was replaced by 0·5 mM-EGTA ($n = 3$). The effects of all concentrations of ACh were reversed by 0·1–1 μM-atropine (data not shown).

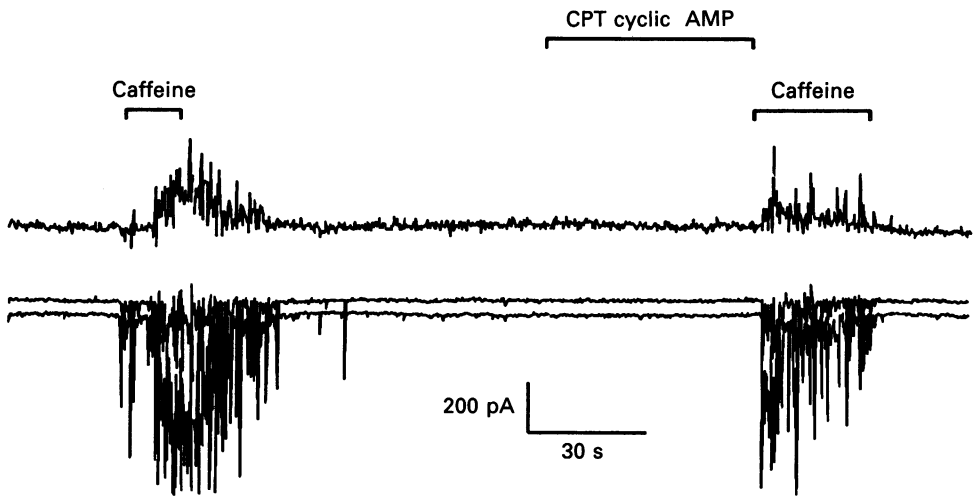

Fig. 3. Pulsatile currents measured at 0 mV (top line), −40 mV (middle line) and −80 mV (bottom line) generated by 1 mM-caffeine, both before and after exposure to 10 μM-CPT cyclic AMP which did not itself induce oscillations.

Figure 2 shows ACh-induced repetitive transient oscillations measured first at a holding potential of −80 mV (*C*) and then at a holding potential of 0 mV (*B*); the data were gathered continuously (no voltage pulses) at a sampling rate of 2 kHz. Compared to Fig. 2, these data show that the pulse protocol did not distort the shape of the transients despite the fact that their period (1–2 s) was close to the effective time resolution (0·5 s) for data collection using pulse protocols. Figure 3 does show that pulse protocol measurements underestimate the extent of the random noise both in the presence and absence of ACh, particularly in the K^+ current.

Caffeine and cyclic AMP

Caffeine is known to be effective in two second messenger pathways. As a phosphodiesterase inhibitor (Sutherland & Rall, 1958) it can cause an increase in cyclic AMP levels, and by acting directly on caffeine-sensitive Ca^{2+} pools, it can increase free Ca^{2+} concentration (Weber & Herz, 1968). Figure 3 shows that 1 mM-caffeine stimulated a repetitive transient current response in both the K^+ and Cl^- currents very similar in appearance to that induced by ACh. When 1 mM-caffeine was the first agonist presented to the cells, oscillations were induced in 60% of cases ($n = 10$). Figure 3 also shows that 10 μM membrane permeable 8-(4-chlorophenothiol) adenosine 3′,5′-cyclic monophosphate (CPT cyclic AMP) did not stimulate any transient or sustained increase in either K^+ or Cl^- current. Both dibutyryl and CPT cyclic AMP were tried at concentrations between 0·1 and 100 μM. In twelve cells derived from seven animals, neither analogue caused any stimulation of either the

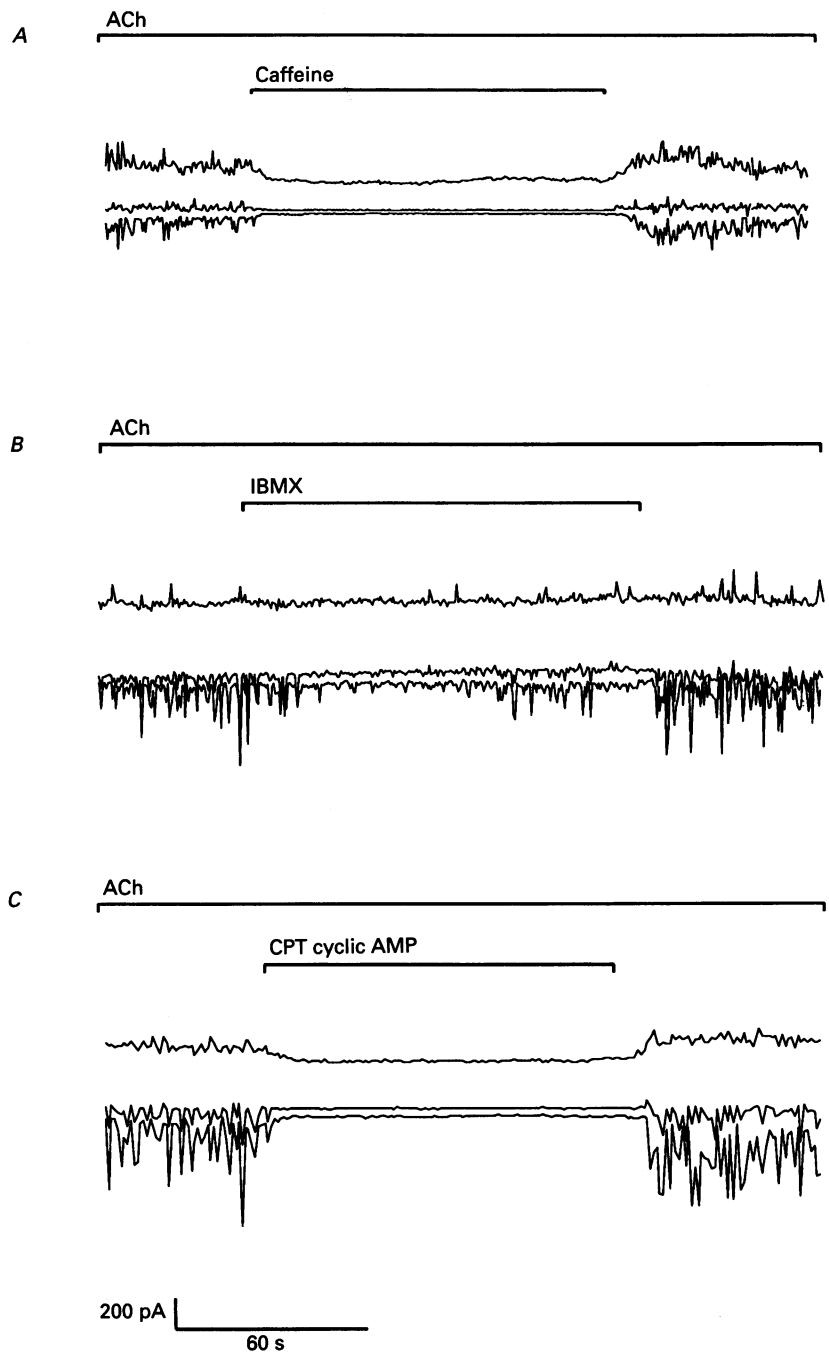

Fig. 4. *A*, pulsatile currents measured at 0 mV (top line), −40 mV (middle line) and −80 mV (bottom line) induced by 20 nM-ACh and inhibited by 10 mM-caffeine. *B*, pulsatile currents measured at 0 mV (top line), −40 mV (middle line) and −80 mV (bottom line) induced by 50 nM-ACh and inhibited by 100 μM-IBMX. *C*, pulsatile currents measured at 0 mV (top line), −40 mv (middle line) and −80 mV (bottom line) induced by 50 nM-ACh and inhibited by 10 μM-CPT cyclic AMP.

K^+ or Cl^- current. 3-isobutyl-1-methylxanthine (IBMX), another xanthine, which can increase cyclic AMP levels (Parsons, Ramkumar & Stiles, 1988), but which is not thought to stimulate Ca^{2+} release from caffeine-sensitive stores also did not stimulate either K^+ or the Cl^- currents. IBMX was applied without stimulatory effect at concentrations between 10 μM and 1 mM ($n = 7$). These observations make it highly unlikely that the caffeine-induced repetitive transient oscillations are mediated by inhibition of phosphodiesterase and increased cyclic AMP levels.

Figure 4*A* shows that a higher dose of caffeine (10 mM) inhibited both the repetitive transient and sustained components of the ACh-induced currents. The inhibitory effect of 10 mM-caffeine was sustained for as long as the caffeine was present and reversed very rapidly after removal of caffeine ($n = 10$). At 1 mM, caffeine also caused some inhibition of ACh-induced transients if it was added subsequent to ACh, but the inhibitory effect of 1 mM-caffeine was often not maintained and in four cases out of eight both the sustained and transient components of the ACh response recovered after 2–3 min in the continued presence of ACh and caffeine. We did not find a concentration of ACh and caffeine at which their stimulatory effects were additive. Figure 4*B* shows inhibition of ACh-induced repetitive transient oscillations by 100 μM-IBMX ($n = 4$). Further evidence that cyclic AMP might be involved in the inhibitory action of caffeine is presented in Fig. 4*C* which shows that 10 μM-CPT cyclic AMP also inhibited ACh-induced repetitive transient oscillations. At this concentration, the inhibition caused by CPT cyclic AMP was sustained in 50% of cases and transient in the other 50% ($n = 6$).

DISCUSSION

Current models of fluid and electrolyte secretion in exocrine glands (Petersen & Gallacher, 1988) require simultaneous activation of both the basolateral K^+ conductance and the luminal Cl^- conductance. A most noticeable feature of almost all of the previous studies in salivary and lacrimal glands is that, while the K^+ current response can be sustained throughout the period of agonist application, the Cl^- current activation is transient and even, on occasion, totally absent (Nishiyama & Petersen, 1974; Putney, 1977; Iwatsuki *et al.* 1985; Morris *et al.* 1987; Sasaki & Gallacher, 1990); this cannot support continued fluid and electrolyte secretion.

In the present study we have shown that lower concentrations of the phosphoinositidase-coupled agonist, ACh (nanomolar as opposed to micromolar) induced a series of brief but repetitive current transients. The significance of these repetitive transient responses was that the Cl^- current activation was often now greater than K^+ current activation and importantly, both K^+ and Cl^- currents were activated throughout the period of agonist stimulation thereby meeting the requirement of concomitant activation to achieve a sustained secretory response. In some cases, we observed a lag in the onset of the ACh response; this only occurred in response to very low agonist concentrations and it is possible that it reflects the rate of $InsP_3$ production in response to stimulation at threshold levels of agonist. We consider that this pattern of repetitive transient activation may well represent the physiological, secretory, response of submandibular acinar cells to phosphoinositidase-coupled agonists.

The only previous report of a repetitive transient current response to a cholinergic agonist in salivary glands is that by Gray in rat parotid acinar cells (1988). Other than the repetitive transient nature of the response, there are few similarities with our findings. In the parotid there was no significant activation of the Cl^- conductance, transients elicited with 10 μM-carbachol were entirely K^+ currents. Furthermore, they had a regular sinusoidal shape with a period of 5–10 s and were abolished within 60 s of removing extracellular Ca^{2+}. These differences could be due to the different species and cell type used or perhaps to the different agonist concentration range (10–250 μM-carbachol).

Although mouse pancreatic acinar cells do not have a Ca^{2+}-activated K^+ conductance, repetitive transient activation of the Ca^{2+}-dependent Cl^- current by cholinergic agonists has been reported (Wakui *et al.* 1989). As in the present study, these transients are driven by Ca^{2+} from internal stores as they persist for long periods in the absence of external Ca^{2+}. Transients have also been induced in pancreatic acinar cells by intracellular application of InsP_3 or Ca^{2+} (Wakui *et al.* 1989; Osipchuk, Wakui, Yule, Gallacher & Petersen, 1990). These findings support the hypothesis that transient and oscillating Ca^{2+} signals arise from repetitive Ca^{2+} release from a Ca^{2+}-sensitive Ca^{2+} pool which is triggered by Ca^{2+} released from an InsP_3-sensitive Ca^{2+} pool. A further support for this hypothesis was the finding that caffeine, which has been shown to promote Ca^{2+}-induced Ca^{2+} release in sarcoplasmic reticulum (Weber & Herz, 1968) and elsewhere (Kuba, 1980; Erlich & Watras, 1988), could evoke Cl^- current transients in the pancreas either in the presence of subthreshold concentrations of ACh (Osipchuk *et al.* 1990) or following Ca^{2+} loading of the cells (Wakui, Osipchuk & Petersen, 1990). In the present study caffeine gave rise to repetitive transient current responses in 60% of cells tested. This is the first report of a stimulatory action of caffeine in the absence of agonist or Ca^{2+} loading in non-excitable cells. This effect of caffeine was not due to phosphodiesterase inhibition since it was not mimicked by either IBMX or cell-permeable cyclic AMP. The transient activation of currents in response to a low dose of cholinergic agonist, the independence of these current transients on extracellular Ca^{2+} and the stimulatory action of caffeine are common features in both pancreas and submandibular cells which point to a similar mechanism of transient generation in both cell types.

It has also been reported that caffeine has an inhibitory effect, blocking both the agonist and InsP_3-mediated Cl^- current responses in both pancreatic acinar cells (Osipchuk *et al.* 1990; Wakui *et al.* 1990) and *Xenopus* oocytes (Parker & Ivorra, 1991). We confirm that caffeine, at concentrations higher than those which stimulate Ca^{2+}-activated currents, does inhibit ACh-induced current transients. We also report, however, that agonist-induced current transients may be inhibited by the phosphodiesterase inhibitor IBMX or by cell-permeable cyclic AMP. It has been shown in a variety of tissues that a cyclic AMP-dependent kinase is able to inhibit InsP_3 production by inhibiting phospholipase C (Bianca, deTogni, Grzeskowiak, Vincenti & di Virgilio, 1986; Puurenen, Lohse & Schwabe, 1987; Madison & Brown, 1988; Neylon & Summers, 1988; Hall, Donaldson & Hall, 1989; Rasmussen, Kelley & Douglas, 1990; Robertson, Bruno & Datta, 1990). This provides a mechanism whereby caffeine and other agents which increase cyclic AMP are able to inhibit agonist-induced Ca^{2+} release and current activation by inhibiting InsP_3 production.

This finding appears in contrast to the previous reports in pancreatic acinar cells (Wakui *et al.* 1990) and *Xenopus* oocytes (Parker & Ivorra, 1991) which showed a substantial inhibition by caffeine of the $InsP_3$-induced current response but at best a very modest inhibition by either cyclic AMP or other phosphodiesterase inhibitors. These separate groups have concluded that the inhibitory effects of caffeine were due to a direct action of caffeine on the Ca^{2+} release consequent to $InsP_3$ generation. Our findings in the submandibular gland do not necessarily contradict the conclusion of these studies, but rather indicate that when receptor activation, rather than direct application of $InsP_3$ provides the stimulus for Ca^{2+} mobilization, caffeine may be able to inhibit $InsP_3$ production via a cyclic AMP pathway in addition to any direct effect on $InsP_3$-induced Ca^{2+} release.

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