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cAMP directly facilitates Ca-induced exocytosis in bovine lactotrophs

Sujit K. Sikdar[1], Robert Zorec[2] and William. T. Mason[1]

[1]*Department of Neuroendocrinology, AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge, CB2 4AT, UK* and [2]*Institute of Pathophysiology, School of Medicine, 61105 Ljubljana, Yugoslavia*

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We have used the whole cell patch clamp technique on single prolactin-secreting bovine lactotrophs to measure plasma membrane capacitance (C_m), an index of membrane surface area, under voltage-clamp during cytosol dialysis with Ca and cAMP. cAMP increased the magnitude and rate of Ca-induced exocytosis (C_m increase) without affecting membrane conductance; however, cAMP had no detectable effect on C_m when intracellular Ca was low. We thus report new evidence that cAMP can facilitate Ca-induced secretion in a synergistic fashion, by acting directly on the secretory apparatus, independently of membrane conductance activation.

Calcium; Exocytosis; Cyclic AMP; Hormone; Secretion; Prolactin; Anterior pituitary

1. INTRODUCTION

The coupling of stimulus to secretion is fundamental to most cellular functions, including secretion of hormones and neurotransmitters. The underlying signal which stimulates exocytotic secretion is a rise in intracellular Ca ions. However, other intracellular messengers may also be important in regulation of exocytosis [1]; in particular, the G-protein family has received wide interest [2,3]. The whole cell patch clamp technique makes it possible to observe directly the membrane events which underly hormone secretion, including both exocytosis and endocytosis due to membrane recovery, by measurement of plasma membrane capacitance (C_m). C_m is an index of membrane surface area; earlier work has shown that in these prolactin-secreting cells it is a measure of a steady state between exocytosis and endocytosis, and is comprised of microscopic fluctuations (increases and decreases) representing fusion and recovery of small membrane packets consistent with the parameters of single secretory granules. In addition, the technique allows the cytosol to be dialysed both with ions of known concentration and putative modulators of secretion [4–7].

Here we have studied the effects of Ca and cAMP, and found that cAMP increased the magnitude and rate of Ca-induced exocytosis (C_m increase) without affecting membrane conductance; in contrast, cAMP had no detectable effect on C_m when intracellular Ca was low. It appears therefore that cAMP facilitate Ca-induced secretion by acting directly on the secretory apparatus but without significantly activating membrane ion channels.

Correspondence address: W.T. Mason, Department of Neuroendocrinology, AFRC Institute of Animal Physiology & Genetics Research, Babraham, Cambridge, CB2 4AT, UK

2. METHODS AND MATERIALS

Bovine pituitary lactotrophs were cultured according to methods described previously [8], and patch clamped in the whole cell mode using well-described methods [9–11]. The recording bath solution contained (in mM): 131.8 NaCl, 5 KCl, 1.8 $CaCl_2 \cdot 2\ H_2O$, 2 $MgCl_2 \cdot 6H_2O$, 0.5 NaH_2PO_4, 5 $NaHCO_3$, 10 Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid)/NaOH, 10 glucose; pH 7.2. The pipette solution contained: 140 K-gluconate, 10 NaCl, $2MgCl_2 \cdot 6H_2O$, 10 Hepes, 0.5 EGTA (ethyleneglycol-bis-(aminoethyl ether)-N,N,N',N'-tetraacetic acid), and different concentrations of Ca-saturated EGTA (Ca-EGTA, 0.5, 1.5 and 3.5 mM), pH 7.2, with Trizma base. EGTA and Ca-EGTA were prepared in 100mM stocks as previously described [12]. Free intracellular Ca concentrations of 150, 450 nM and 1 μM Ca were estimated using the equation:

$$[Ca^{2+}] = [\text{Ca-EGTA}]/[\text{EGTA}] \times K_d$$

where K_d = dissociation constant = 0.15 μM [13]. The Ca-free internal solution contained 10 mM EGTA. The cAMP and dbcAMP were obtained from Sigma, all other reagents from Sigma and Aldrich, highest purity available. The cells were voltage clamped at a holding potential of −70 mV. The reversal potential of the whole-cell current was −50 mV. Membrane capacitance was recorded using a two-phase lock-in amplifier (sinusoidal frequency, 1600 Hz, 1 mV peak-peak) incorporated into a patch-clamp amplifier [14]. The plots of the passive cell parameters: access conductance (G_a), parallel combination of leak and membrane conductance (G_m) and membrane capacitance (C_m) were derived by a computer-aided reconstruction using an analogue/digital converter (CED 1401, Cambridge, UK) to record the signals in digital format on the computer hard disc. The dc current (1 − 10 Hz, −3dB) and the real and imaginary admittance signals (0 − 1 Hz, −3dB) were plotted on a chart recorder (Gould 2400). Recordings were made at room temperature which was 22°C. The software (John Dempster, University of Strathclyde) also utilized holding potential and reversal potential for calculating the passive cell parameters [15,16].

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3. RESULTS AND DISCUSSION

The top trace in Fig. 1A shows the change of cell membrane capacitance (C_m) in a cell dialyzed with 450 nM Ca. The initial flat portion of the C_m trace of zero C_m indicates the cell-attached configuration, the sharp upward deflection of the C_m trace from base line indicates the point where the whole-cell configuration was attained, and the C_m value at that instant relates to the resting C_m ($C_{m.rest}$) due to inclusion of the whole-cell C_m in the electrical circuit. The change in C_m following this jump, however, relates to the subsequent cellular events regulating the fusion of secretory granules with the plasma membrane, and thereby is a measure of cellular secretory response. In other experiments also performed at room temperature, we observed that the gradual increase in C_m was composed of microscopic increases and decreases in C_m which were attributable to exocytosis and endocytosis, respectively. The lower C_m trace from a different cell shows the C_m change following dialysis after patch rupture, with a similar concentration of intracellular Ca but in the presence of 0.1 mM cAMP and 1 mM ATP (cAMP/ATP). ATP was included in the dialysate as a phosphorylating source. The increase in C_m from rest is larger in the presence of cAMP/ATP compared to control (see Fig. 1 legend). The traces in Fig. 1B are from two different cells dialyzed with a higher concentration of intracellular Ca (1 μM). Again, it is evident from the traces that the C_m change was larger in the cell dialyzed additionally with cAMP/ATP (lower trace) than in the cell dialyzed with intracellular Ca alone (upper trace).

The increase in C_m promoted by cAMP could be due to action on membrane ionic channels to increase intracellular Ca or, alternatively, it might result from an effect on cytosolic structures involved in hormone secretion, or a combination of both factors. During the course of an experiment, membrane current (low pass filtered <10 Hz, -3 dB) tended to increase in around 70% of cells (up to 7-fold), probably due to degradation of the membrane-pipette seal (Fig. 1C,D). However, a transient increase of the leakage current through the membrane-pipette seal had no effect on the increase of C_m (Fig. 2A). In 13 such studied cells, the C_m signal was affected by a transient increase in leakage current in one cell only. A similar observation was reported in mast cells [15]. Moreover, no correlation was observed between the normal increase in C_m and the estimated increase in membrane conductance (G_m) in control and treated cells (Fig. 2B). There was no detectable change in membrane current in control and cAMP dialyzed cells during the initial phase of C_m rise at the holding potential of -70mV. The inward current amplitude 1 min after patch rupture was not significantly different in control with 450 nM Ca (4.2 ± 1.0 pA, mean SEM, $n=7$) versus the same Ca concentration in the presence of cAMP/ATP (4.7 ± 1.0 pA, $n=5$). The corresponding values for 1 μM Ca were 9.1 ± 2.5 pA, $n=7$ (control) and 6.6 ± 1.1 pA, $n=9$ (+cAMP/ATP). This suggests that the effects of cAMP/ATP on C_m observed are probably directly on the cytosolic structures involved in hormone secretion, and are in the main not due to indirect effects on membrane conductance. However, one cannot exclude a possible effect on calcium channels, because very small currents of calcium could contribute significantly to intracellular calcium homeostasis [31].

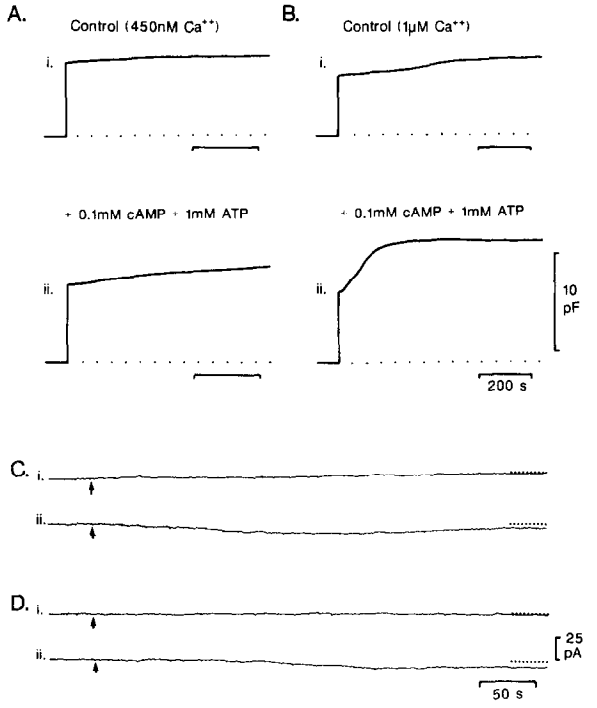

Fig. 1. C_m measurements in cells dialyzed with 450 nM Ca (A) and 1 μM Ca (B). The upper traces show C_m changes in cells dialyzed with Ca alone (Control) while the lower traces are from cells dialyzed with 0.1 mM cAMP and 1 mM ATP (cAMP/ATP) in the presence of Ca. Note the larger increase in C_m from rest (see text) in the cAMP/ATP dialyzed cells compared to Ca alone. Also note the faster rise time for the cell in B_{ii} compared with B_i. The secretory response (see text) for the cells shown in A_i, A_{ii}, B_i and B_{ii} were 7.8%, 23.5%, 25% and 73.7%, respectively, while the max.slope/$C_{m.rest}$ values (see text) for the cells shown in B_i and B_{ii} were 0.086 and 0.655 (100 s^{-1}), respectively. The recordings are from 4 different cells. $C_{i,ii}$ and $D_{i,ii}$ represent membrane current recordings for the cells shown in $A_{i,ii}$ and $B_{i,ii}$, respectively. The arrows indicate the initiation of cytosol dialysis. The dotted lines show the zero level.

Fig. 3A summarizes the effects on intracellular cAMP/ATP on the secretory response at different intracellular Ca concentrations. With 10 mM EGTA in the dialysate the secretory response was very small and unaffected by intracellular cAMP/ATP. Intracellular Ca concentrations of 150 and 450 nM were not sufficient to induce a significant secretory response, although the response with intracellular cAMP/ATP appeared to be larger in the 150 nM Ca group than with 150 nM Ca alone. The facilitatory effect of

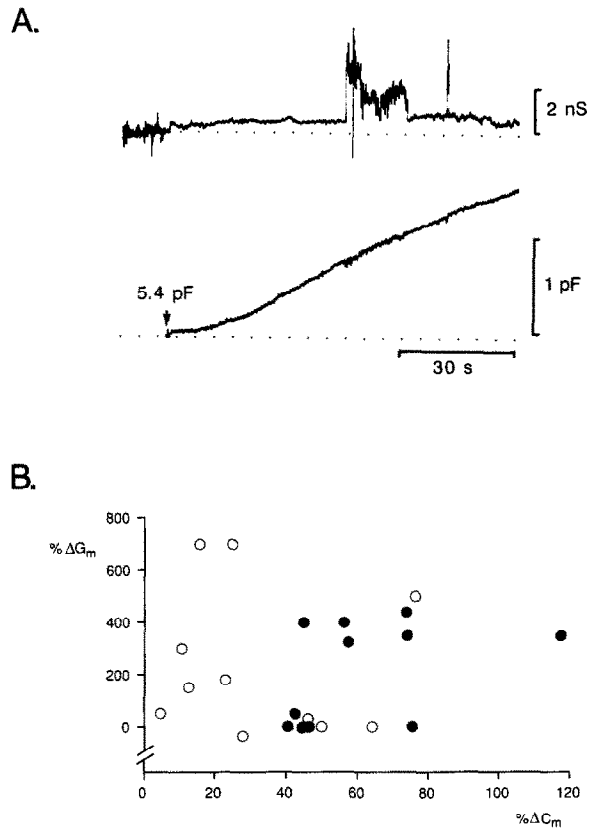

Fig. 2. (A) The effect of a transient leak (membrane-pipette seal breakdown) observed as an increase in membrane conductance (G_m) on the time course of the C_m increase. Membrane conductance (top trace) was estimated from membrane current and the driving potential (holding potential - reversal potential). Reversal potential was determined to be −50 mV in these cells. The cell was clamped at −70 mV while the cytosol was dialysed with pipette solution containing (in mM) 3.5 Ca-EGTA, 0.5 EGTA (free Ca^{2+} concentration = 1 μM), 0.1 mM cAMP and 1 mM ATP. Arrow denotes the start of cytosol dialysis. Resting C_m of 5.35 pF was digitally subtracted from the C_m signal (lower trace). (B) Relationship between maximal increase in C_m and G_m relative to resting C_m and G_m following cytosol dialysis by pipette solutions with (in mM): 0.5 EGTA, 3.5 CaEGTA (o); and 0.5 EGTA, 3.5 CaEGTA, 0.1 cAMP and 1 ATP (●).

cAMP/ATP on the cells with intracellular Ca levels of 450 nM was, however, significant compared to control ($P<0.05$). Inclusion of ATP (1 mM) alone did not have a significant effect on the secretory response in the presence of 450 nM Ca ($P>0.05$, $n=4$). Intracellular Ca levels of 1 μM induced exocytosis on its own [17–19]. Inclusion of cAMP/ATP (0.1 mM/1 mM) in the dialysate containing 1 μM Ca significantly potentiated the secretory response when compared to 1 μM Ca alone ($P<0.05$). Thus cAMP/ATP does not induce a secretory response in the absence of Ca, but enhances the Ca-induced secretory response.

We further examined the effect of intracellular cAMP by utilizing the membrane permeable cAMP analogue, N^6,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate (dbcAMP). Application of dbcAMP to the bath (10 mM) induced a faster rise in C_m to a higher steady state but there was no effect on the G_m trace (Fig. 3B, $_i$; $n=3$). Interestingly, a similar effect was observed in cells dialyzed with added 0.1 mM cAMP ($n=2/3$), presumably due to further increase in intracellular free cAMP concentration. Applications of dbcAMP in cells dialyzed with 10 mM EGTA and 1 mM ATP, however, failed to produce an increase in C_m (Fig. 3B $_{ii}$; $n=2$). These results further suggest that the cAMP effect is expressed through a cytosolic Ca-dependent process, and is consistent with observations in cells dialysed with 10 mM EGTA and cAMP/ATP (Fig. 3A).

In addition to increasing the secretory response, cAMP/ATP also affected the time course of the response. In Fig. 1B, for instance, the C_m change is faster in the presence of cAMP/ATP (lower trace) compared to control (upper trace). The time course of the secretory response was quantified by determining the maximal slope (dC_m/dt, pF/100 s), and normalizing the values with $C_{m.rest}$ for individual cells (max.slope/$C_{m.rest}$). These parameters were compared in the cells dialyzed with 1 μM Ca alone and in the presence of added cAMP/ATP where the rate of C_m rise was greater ($P<0.005$, Fig. 4). It has previously been shown that fusion of secretory granules with the plasma membrane is an event occuring in less than a second in chromaffin cells [10], pancreatic acinar cells [20], mast cells [21] and in bovine lactotrophs (Zorec, Sikdar and Mason, in press). Therefore, the relatively slow rise in C_m following cytosol dialysis is rate-limited by a much slower process(es) [19,22], which is (are) affected by the presence of cAMP/ATP.

Our results provide evidence of a direct facilitatory action of cAMP/ATP on the Ca-induced C_m increase in lactotrophs which is most probably not mediated via the modulation of ionic channels. It cannot be excluded that addition of cAMP/ATP may have had an effect on calcium channels and calcium influx, because small changes in calcium currents can lead to significant changes in cytosolic calcium activity [31]. However, such an effect is unlikely, as under conditions where the calcium gradient is increased (Fig. 3B$_{ii}$) by lowering of intracellular calcium with 10 mM EGTA, despite the fact that the spontaneous increase in C_m is supported by a tonic influx of calcium [34], addition of dbcAMP does not accelerate the C_m increase.

Two parameters of the secretory response involved in bovine prolactin secretion, measured as membrane capacitance (surface area) changes, have been found to be dependent on cAMP: (1) the maximal increase in C_m and (ii) the time-course of Ca-induced C_m increase. The Ca-induced C_m increase is proportional to the balance between exocytotic addition and endocytotic removal of membrane [2]. It is likely that the larger C_m increase seen with intracellular cAMP/ATP could result from enhancing the exocytotic addition or decreasing endocytotic removal of membrane, or a combination of

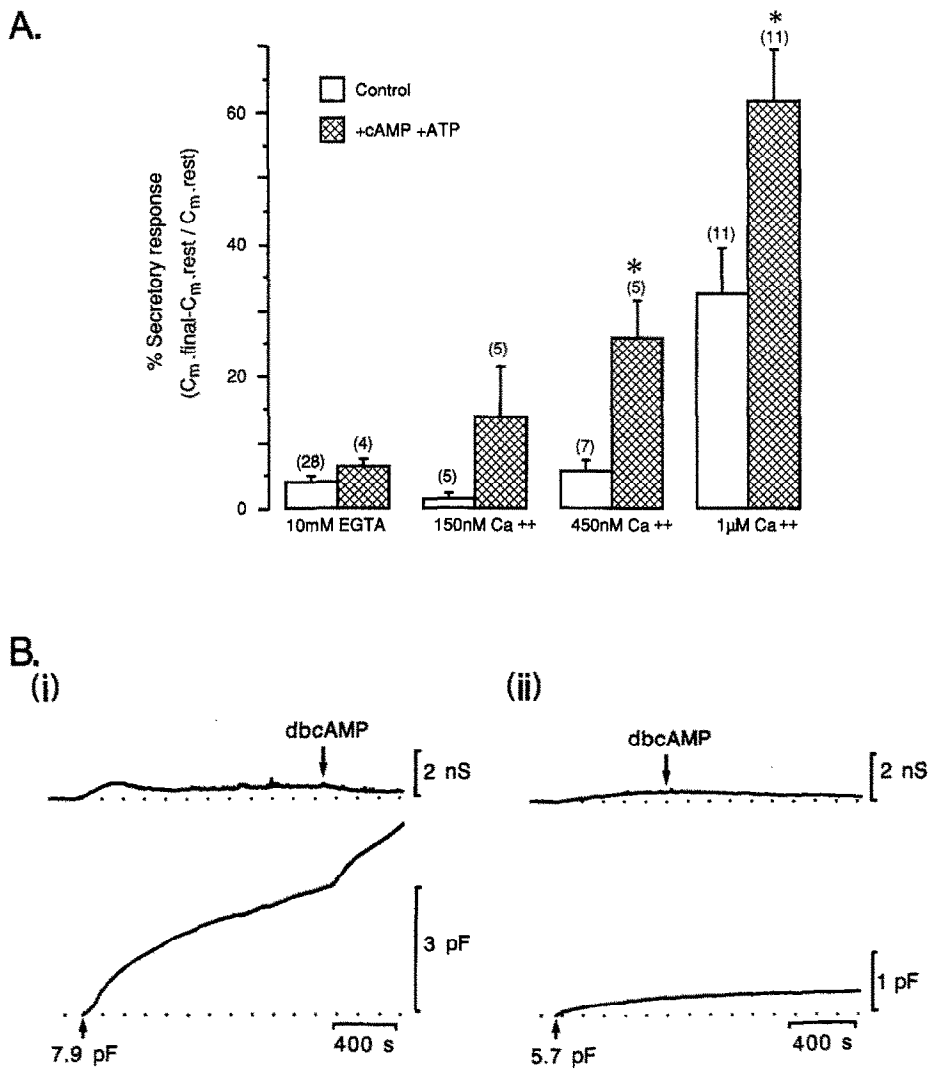

Fig. 3. (A) The effect of intracellular cAMP/ATP (0.1 mM/1 mM) on the secretory responses induced by different concentrations of intracellular Ca. To normalize the secretory response of our recordings we determined the C_m value 8 min after $C_{m.rest}$ ($C_{m.final}$). The secretory response was expressed as the percentage ($C_{m.final} - C_{m.rest}$)/$C_{m.rest}$. The values are mean SEM; n number of observations. Statistical test: the Welch's test for Behren's Fisher problem [34]. $^*P<0.05$. (B) Effects of dibutyryl cAMP on C_m. The membrane permeable analogue of cAMP was applied as a concentrated bolus (20 μl of 0.5 M dbcAMP applied 10–15 mm upstream of the recording pipette: bath volume 1 ml) at the point indicated by arrow to achieve the concentration of 10 mM. The C_m plots shown were obtained by digital subtraction of the resting initial C_m value ($C_{m.rest}$, indicated by arrow) from the C_m plot, to show the time course of secretory response. The cell in (i) was dialyzed with 450 nM Ca and 1 mM ATP while the cell in (ii) was dialyzed with 10 mM EGTA and 1 mM ATP.

both, since we have noted these capacitance changes – even measured at room temperature – to be comprised of prominent increases and decreases in C_m indicating that both exo- and endocytosis are occurring. However, since addition of cAMP/ATP to low intracellular Ca pipette solutions did not decrease C_m (e.g. see Fig. 3B$_{ii}$), nor the secretory response (Fig. 3A), it is more likely that the increased Ca-induced secretory response in the presence of cAMP/ATP is due to facilitation of processes linked to exocytosis rather than endocytosis. This explanation would be consistent with experimental evidence in adrenal chromaffin cells, where endocytosis was suggested to be a Ca-independent process [23].

Increased secretory response and the faster time-course of C_m change by cAMP/ATP may involve increased availability of secretory granules at the fusion site. This could be due to increased probability of successful fusion of a docked granule. It could also be due to facilitated translocation of granules across cytosolic barriers, such as the cytoskeleton [24,25] or endoplasmic reticulum [26], to the fusion site. Reorganisation of these strucures may be cAMP dependent, as demonstrated in bovine anterior pituitary [27]. Association of a secretory granule membrane protein with the cytoskeletal elements may be involved, such as synapsin I [28,29]. Phosphorylation of secretory

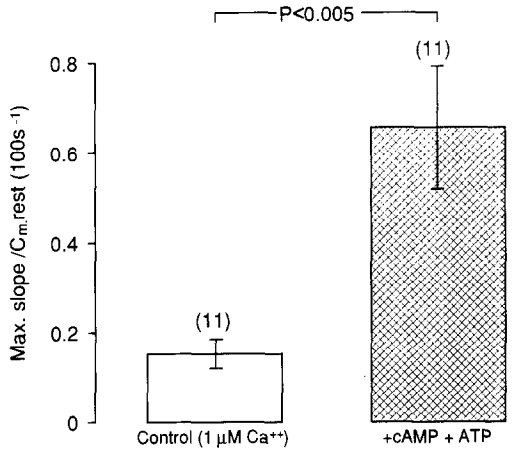

Fig. 4. Facilitation of the time-course of exocytosis in cAMP/ATP dialysed cells containing 1 μM Ca. Maximal slope (dC_m/dt, pF/100s) values were obtained by drawing a tangent (by hand) at the point showing the steepest rise in the C_m trace and were normalized with $C_{m.rest}$ of individual cells.

granule membrane-associated proteins by cAMP has been reported in anterior pituitary cells [30], and very recently the regulatory subunit of cAMP-dependent protein kinase has been identified in secretory granules using a monoclonal antibody to the regulatory subunit combined with immunogold labelling [31].

Studies on adrenal chromaffin cells have shown that proteins such as calpactin or p36 [32] and chromaffin granule binding protein [33] are essential for exocytosis. The fusion proteins could play a regulatory role in phosphorylation-dependent secretion. Such a process could underlie our findings and is consistent with the greater increase in C_m in cells dialyzed with cAMP/ATP.

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