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Cholinergic receptors regulate a voltage-insensitive but Na^+-dependent calcium influx pathway in salivary acinar cells

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The calcium-sensitive fluorescent probe, quin 2, was employed to investigate the cholinergic receptor regulation of cytosolic free calcium concentration ($[Ca^{2+}]_i$) in isolated rat submandibular acinar cells. Cholinergic receptor stimulation results in a marked and sustained elevation $[Ca^{2+}]_i$. The major component of this response is shown to be due to activation of a calcium influx pathway. There is no evidence in K^+-depolarized cells of any voltage-gated calcium influx. Acetylcholine failed to activate the calcium influx pathway in cells bathed in Na^+-free solutions. The cholinergic receptors thus regulate a voltage-insensitive but Na^+-dependent transport system for calcium entry in submandibular acinar cells.

Acetylcholine; Ca^{2+} influx; Na^{2+} dependence; Quin 2; (Salivary gland, Submandibular gland)

1. INTRODUCTION

In secretory cells, as in a wide range of biological systems, an essential component of the cells response to neurotransmitters is an influx of calcium from the extracellular to intracellular fluid [1,2]. It is the consequent elevation in cytosolic free calcium concentration which promotes the cellular response. In excitable cells this calcium influx can generally be shown to be due to the activation of voltage-dependent, calcium-selective ion channels in the membranes [3,4]. In non-excitable cellular systems, such as salivary acinar cells, the pathway for calcium influx and the manner in which it is regulated are not at all defined [2,5]. Here, the intracellular calcium-sensitive, fluorescent indicator, quin 2 [6] is employed to monitor the time course and amplitude of the change in cytosolic free calcium concentration ($[Ca^{2+}]_i$) induced in salivary (submandibular) acinar cells by the neurotransmitter acetylcholine (ACh). The elevation in $[Ca^{2+}]_i$ induced by ACh is shown to be due to (i) release of calcium from intracellular stores, and (ii) (the major component) influx of calcium from the extracellular to intracellular fluid. The properties of the receptor-regulated calcium influx pathway are investigated.

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2. MATERIALS AND METHODS

Submandibular glands were excised from adult, male rats and acini enzymatically isolated by hyaluronidase and collagenase digestion [5]. The control incubation media contained (mM): NaCl, 140; KCl, 4.5; $CaCl_2$, 2.5; $MgCl_2$, 1.13; glucose, 10; Hepes, 10. Solutions were titrated to pH 7.4

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and gassed with 100% O_2. In the Ca^{2+}-free solutions $CaCl_2$ was omitted and 0.1 mM EGTA added. Na^+-free solutions were prepared by substituting all Na^+ for either K^+ or the large impermeant cation, *N*-methyl-D-glucamine ($NMDG^+$) [5,7]. The enzymatically isolated acini were incubated in control, Hepes-buffered, solutions containing 2% BSA and 20 μM quin 2 amoxymethyl ester (Amersham, England) for 30 min at 37°C. The acini were washed, centrifuged and resuspended in normal or test solutions for 15 min prior to experimentation. Fluorescence was measured at 30°C in a Perkin-Elmer LS-5 fluorometer (excitation 339 nm, emission 492 nm) with continuous stirring. None of the experimental protocols resulted in any significant (< 1%) change in the autofluorescence of the cell suspensions. Fluorescence due to external quin 2 was determined in each experiment using Mn^{2+} and DTPA (diethylenetriampentaacetic acid) quench protocols (see [8]). At the end of each experiment the cells were disrupted by sonication, F_{max} and F_{min} determined and $[Ca^{2+}]_i$ calculated according to Tsien et al. [6]. $[Ca^{2+}]_i$ is expressed in nM ± SE. Statistical significance was assessed by Student's *t*-test and equality of variance tested by variance ratio or F-test.

3. RESULTS

The resting cytosolic free calcium concentration ($[Ca^{2+}]_i$) in the submandibular acinar cells was 77 ± 7 nM (n = 21) with a range of 38–112 nM. The only other study employing quin 2 in salivary acinar cells reports the higher resting value of 160 nM for parotid acinar cells [9]. ACh stimulation results in a dose-dependent increase in $[Ca^{2+}]_i$. The effect was maximal at 10^{-5} M ACh and this concentration was employed throughout this study. Fig.1 shows a typical experiment. ACh stimulation resulted in a rapid rise in $[Ca^{2+}]_i$, to a mean of 279 ± 27 nM (n = 14), within the first minute of stimulation. The $[Ca^{2+}]_i$ then declined to a lower, but still elevated, level that was sustained throughout the period of ACh application. The effect of ACh on $[Ca^{2+}]_i$ were totally blocked or reversed by application of the muscarinic cholinergic antagonist atropine (10^{-5} M) (see fig.1). In the absence of extracellular calcium (i.e. no added calcium, 0.1 mM EGTA) the resting

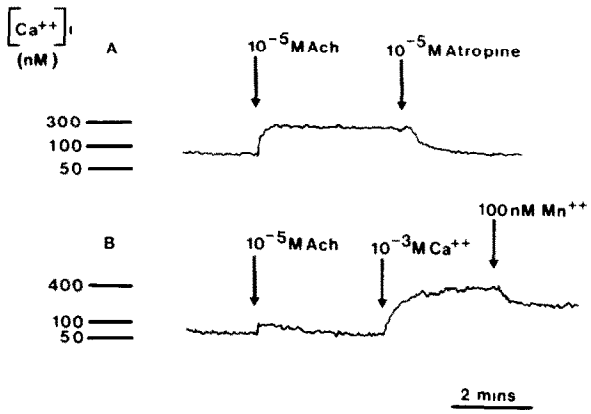

Fig.1. Changes in $[Ca^{2+}]_i$ (quin 2 fluorescence) induced by acetylcholine (ACh, 10^{-5} M) in isolated submandibular acinar cells. (A) Response to ACh of acinar cells bathed in normal control solution containing 2.5 mM calcium. There is a rapid and sustained elevation in $[Ca^{2+}]_i$ that is only restored to pre-stimulus levels upon reintroduction of the muscarinic cholinergic receptor antagonist atropine (10^{-5} M). (B) Effect of ACh (10^{-5} M) on $[Ca^{2+}]_i$ in acinar cells bathed in calcium-free solution. ACh evokes only a small transient elevation in $[Ca^{2+}]_i$. The reintroduction of calcium (1.3 mM) to the extracellular solution, in the continued presence of ACh, results in a sustained elevation of $[Ca^{2+}]_i$. The Mn^{2+} (100 nM) is added to displace calcium from extracellular quin 2. Such Mn^{2+}, DTPA quench protocols were employed in all experiments (see [8]). All values (nM) given for $[Ca^{2+}]_i$ in the text are corrected for this extracellular component.

$[Ca^{2+}]_i$ was 64 ± 10 nM (n = 5) and ACh stimulation resulted in only a small and transient elevation in $[Ca^{2+}]_i$, maximal at 86 ± 5 nM. The changes in $[Ca^{2+}]_i$ induced by ACh are thus very largely dependent on the presence of extracellular calcium. In calcium-free solutions, in the continued presence of ACh, the reintroduction of calcium to the bathing solution (1.13 mM) results in a rapid and sustained increase in $[Ca^{2+}]_i$ (fig.1). This elevation in $[Ca^{2+}]_i$ upon reintroduction of calcium was totally blocked in each of three experiments by prior application of atropine (10^{-5} M). The elevation in $[Ca^{2+}]_i$ induced by ACh is thus predominantly mediated by the activation of a calcium influx pathway.

Two different protocols were employed to test for any effect of voltage on the calcium influx pathway (fig.2). Acinar cells were bathed in con-

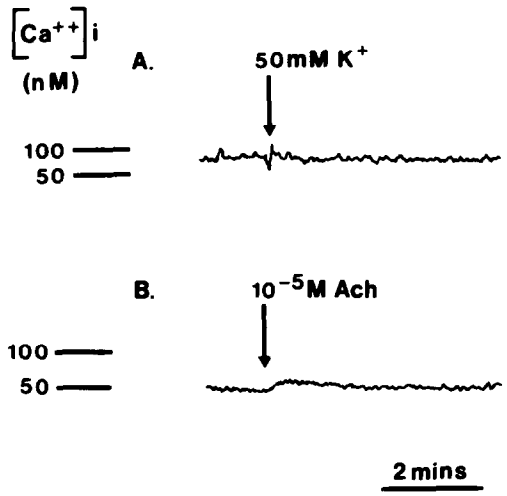

Fig.2. (A) Cells are bathed in a control (NaCl) solution containing 2.5 mM calcium. At the point indicated 50 mM KCl is added to the extracellular solution. There is no change in $[Ca^{2+}]_i$ in response to the acute K^+ depolarization. (B) Cells are incubated from outset in an Na^+-free, 145 mM KCl solution (i.e. K^+-depolarized). ACh (10^{-5} M) is applied at the point indicated. ACh stimulation is associated with a small, transient elevation in $[Ca^{2+}]_i$.

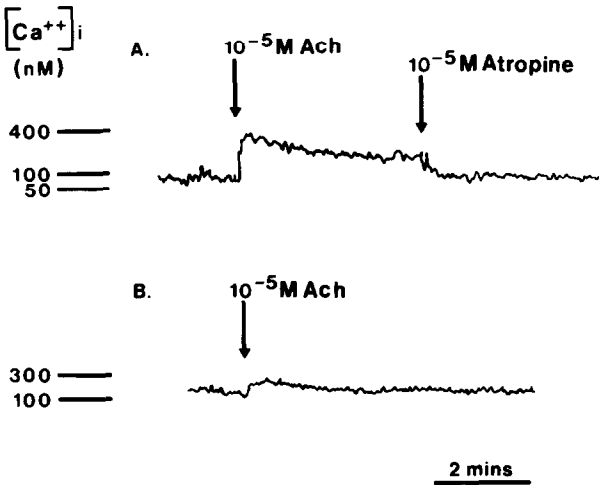

Fig.3. The cells employed in the experiments shown in A and B were derived from the same enzymatic digestion. (A) These cells have been suspended in normal (NaCl), calcium-containing (2.5 mM) solution. The control response to ACh (10^{-5} M) is shown for this cell preparation. (B) These cells have been suspended in Na^+-free ($NMDG^+$-substituted) solution 15 min prior to recording. It is seen that in the Na^+-free ($NMDG^+$) solution ACh evokes only a transient rise in $[Ca^{2+}]_i$.

trol solutions containing 2.5 mM calcium. The cells were acutely depolarized by addition of 50 mM KCl to the extracellular solution. This did not result in any change in $[Ca^{2+}]_i$ (fig.2, upper trace). In the second series of experiments the cells were incubated in a buffered solution containing 2.5 mM calcium but with all Na^+ replaced by K^+ (i.e. 145 mM KCl). In this situation it has been shown that the acinar cell membrane potential is 0 mV [10]. The resting $[Ca^{2+}]_i$ in this situation was 47 ± 8 nM (n = 7). The application of ACh to the cells bathed in this high K^+ solution resulted in only a small transient increase in cytosolic calcium (maximal $[Ca^{2+}]_i$ = 73.4 ± 10 nM). The transient effect of ACh stimulation in the K^+-depolarized cells was qualitatively and quantitatively very similar to that in calcium-free media.

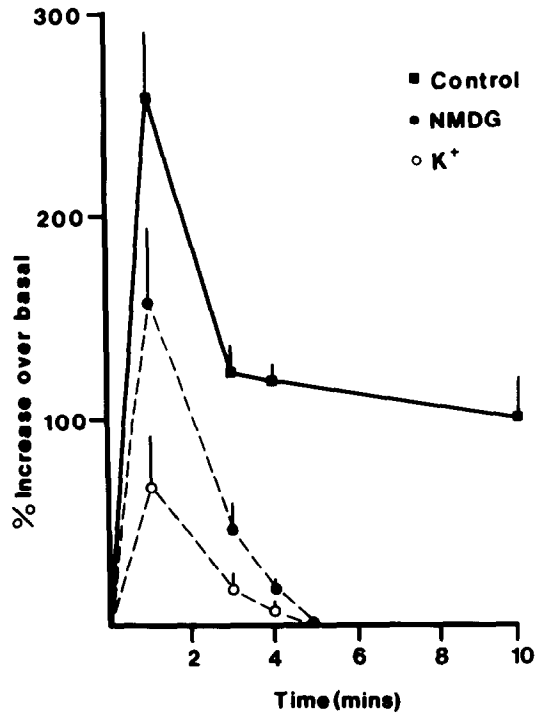

Fig.4. Time course and amplitude of the changes in $[Ca^{2+}]_i$ in submandibular acinar cells induced by ACh (10^{-5} M). The change in $[Ca^{2+}]_i$ is expressed as a % increase over basal ± SE. The effect of ACh is shown for control and the two Na^+-free solutions. The difference between the control and the Na^+-free experiments is highly significant ($p < 0.001$) for all points after the first minute expressed either as % increases over basal or as absolute values (nM).

To test whether the lack of response to ACh of the cells bathed in the high K^+ solution was due to membrane depolarization or the consequence of incubation in an Na^+-free solution the experiments were repeated using an alternative, non-depolarizing, replacement for Na^+. When the large impermeant cation $NMDG^+$ is substituted for Na^+ the acinar cells retain a normal membrane potential [5]. The resting $[Ca^{2+}]_i$ of the $NMDG^+$-bathed cells was 78 ± 10 nM (n = 10), almost identical to the control situation. In this Na^+-free solution (as for the K^+ substituted solutions) ACh application resulted in only a transient elevation in $[Ca^{2+}]_i$ (fig.3). The transient response was significantly ($p < 0.01$) larger in the $NMDG^+$ (maximal increase to 185 ± 23 nM, n = 10) than in the K^+-substituted Na^+-free solution. The sustained component (5 min after introduction of ACh) is however abolished in both of the Na^+-free solutions.

Fig.4 summarizes the data from the experiments detailed above and shows the time course and magnitude of the changes in $[Ca^{2+}]_i$ induced by ACh in the control and test conditions.

4. DISCUSSION

It has long been recognised that salivary secretion is dependent on the presence of calcium in the extracellular fluid [11]. The present study directly demonstrates the importance of calcium influx from the extracellular to intracellular fluid in sustaining the elevation in $[Ca^{2+}]_i$ that is the consequence of cholinergic (muscarinic) receptor activation. In the absence of extracellular calcium ACh stimulation results in a small, transient elevation in $[Ca^{2+}]_i$. The full, sustained response is restored upon reintroduction of calcium to the extracellular fluid in the presence, but not absence, of the agonist. The effect of ACh on $[Ca^{2+}]_i$ is thus predominantly mediated by activation of a calcium influx pathway. The effect of ACh on calcium influx is not mimicked by K^+ depolarization of the acinar cells. There is then no evidence of any voltage-activated calcium influx pathway in submandibular acinar cells.

When the acinar cells are incubated in either of the two Na^+-free solutions ($NMDG^+$ or K^+ substituted for Na^+) the cells respond to ACh with only a transient elevation in $[Ca^{2+}]_i$. It appears then that the calcium influx pathway, but not release of intracellular calcium, is refractory to ACh. This observation is in agreement with a recent electrophysiological study from this laboratory which reports that the ability of ACh to stimulate calcium-activated K^+ channels in intact submandibular acinar cells is abolished in Na^+-free solutions [5]. In the present study as in that of Gallacher and Morris [5] the effect of Na^+-free media takes some 5–10 min to develop. This most probably indicates that the refractoriness of the calcium influx pathway to ACh does not reflect an acute dependence on extracellular Na^+, but is more likely to be due to some change in intracellular environment resulting from Na^+ withdrawal. There are two likely consequences upon removal of extracellular Na^+. The first is a change in $[Na^+]_i$. The acinar cells will be depleted of intracellular Na^+ and the elevation in $[Na^+]_i$ that is normally associated with ACh stimulation [12,13] will be abolished. Secondly Na^+/H^+ exchange will be blocked. This could result in an intracellular acidification in the resting cells and/or prevent the intracellular alkalinization that is reported during secretory stimulation [14]. It is possible that the calcium influx in salivary acinar cells is regulated by either $[Na^+]_i$ or $[H^+]_i$ (see [15,16]).

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