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Evidence for Two Modes of Ca^{2+} Entry Following Muscarinic Stimulation of a Human Salivary Epithelial Cell Line

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Summary. We have investigated muscarinic receptor-operated Ca^{2+} mobilization in a salivary epithelial cell line, HSG-PA, using an experimental approach which allows independent evaluation of intracellular Ca^{2+} release and extracellular Ca^{2+} entry. The carbachol (Cch) dose response of intracellular Ca^{2+} release indicates the involvement of a single, relatively low-affinity, muscarinic receptor site ($K_{0.5} \cong 10$ or 30 μM, depending on the method for $[Ca^{2+}]_i$ determination). However, similar data for Ca^{2+} entry indicate the involvement of two Cch sites, one consistent with that associated with Ca^{2+} release and a second higher affinity site with $K_{0.5} \leq 2.5$ μM. In addition, the Ca^{2+} entry response observed at lower concentrations of Cch (2.5 μM) was completely inhibited by membrane depolarization induced with high K^+ (>55 mM) or gramicidin D (1 μM), while membrane depolarization had little or no effect on Ca^{2+} entry induced by 100 μM Cch. Another muscarinic agonist, oxotremorine-M (100 μM; Oxo-M), like Cch, also induced an increase in the $[Ca^{2+}]_i$ of HSG-PA cells (from 72 ± 2 to 104 ± 5 nM). This response was profoundly blocked (~75%) by the inorganic Ca^{2+} channel blocker La^{3+} (25–50 μM) suggesting that Oxo-M primarily mobilizes Ca^{2+} in these cells by increasing Ca^{2+} entry. Organic Ca^{2+} channel blockers (verapamil or diltiazem at 10 μM, nifedipine at 1 μM), had no effect on this response. The Oxo-M induced Ca^{2+} mobilization response, like that observed at lower doses of Cch, was markedly inhibited (~70–90%) by membrane depolarization (high K^+ or gramicidin D). At 100 μM Cch the formation of inositol trisphosphate (IP_3) was increased 55% above basal levels. A low concentration of carbachol (1 μM) elicited a smaller change in IP_3 formation (~25%), similar to that seen with 100 μM Oxo-M (~20%). Taken together, these results suggest that there are two modes of muscarinic receptor-induced Ca^{2+} entry in HSG-PA cells. One is associated with IP_3 formation and intracellular Ca^{2+} release and is independent of membrane potential; the other is less dependent on IP_3 formation and intracellular Ca^{2+} release and is modulated by membrane potential. This latter pathway may exhibit voltage-dependent gating.

Key Words Ca^{2+} entry · Ca^{2+} mobilization · salivary epithelia · muscarinic · cholinergic · membrane potential

Introduction

Many hormones and neurotransmitters exert their effects by increasing the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$)[1] of target cells. This increase in $[Ca^{2+}]_i$ results from both the release of Ca^{2+} from an intracellular store and from Ca^{2+} entry across the plasma membrane [e.g., 2, 7, 26]. It is now generally accepted that intracellular Ca^{2+} release is primarily due to the generation of IP_3 by the activation of cell-surface receptors [2, 4], but the mechanisms underlying receptor-operated Ca^{2+} entry are not clear [2, 4, 7, 26].

It was initially thought that receptor-operated Ca^{2+} entry followed Ca^{2+} release [26]. In this regard, several laboratories have provided evidence suggesting a role for IP_3 and its metabolites, in particular IP_4 (inositol tetrakisphosphate), in Ca^{2+} entry [7, 22]. However, in a recent review, Exton summarized evidence indicating that Ca^{2+} entry can occur before Ca^{2+} release or, in some cases, in the absence of Ca^{2+} release. Based on these and other observations, he proposed that G proteins rather than IP_3 metabolites, may be involved in receptor-operated Ca^{2+} entry [7]. Similarly, recent studies by Merritt, Jacob and Hallam [18] argue strongly against the existence of a receptor-operated $[Ca^{2+}]_i$-activated Ca^{2+} entry mechanism.

Ca^{2+} entry pathways across the plasma membrane, so-called Ca^{2+} channels, may be voltage dependent or voltage independent. Ca^{2+} channels are termed voltage dependent when their opening and closing (gating) is regulated by membrane potential; typically these channels open in response to mem-

[1] *Abbreviations:* Cch, carbachol; IP_3, inositol trisphosphate; IP_4, inositol tetrakisphosphate; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; EMEM, Eagle's minimal essential medium; DTPA, diethylenetriaminepentaacetic acid; BSS, balanced salt solution; quin-2/AM, the acetoxymethyl ester of quin-2; fura-2/AM, the acetoxymethyl ester of fura-2; Oxo-M, oxotremorine-M acetate; BSA, bovine serum albumin; EGTA, ethylene *bis* (oxyethylenenitrilo) tetraacetic acid; and HEPES, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid.

brane depolarization. Receptor-operated Ca^{2+} entry pathways are generally considered to be voltage independent [6, 17, 27]. However, Ca^{2+} influx via receptor-operated pathways has been shown to be affected by membrane potential in a number of systems. For example, high K^+ attenuates the sustained Ca^{2+} increase induced by Cch in rat parotid acinar cells [20]. Similar phenomena have also been observed in WEHI-231 B lymphoma cells [16], T lymphocytes [8, 24], human neutrophils [34] and J774 macrophages [9]. Conversely, voltage-dependent Ca^{2+} channels may be modulated by hormones, neurotransmitters, and other agents [27]. For example, the activation of muscarinic receptors selectively inhibits high K^+-induced Ca^{2+} entry in rat sympathetic neurons [36]. This modulation of voltage-dependent Ca^{2+} channels may also be mediated by G proteins [14]. Whether these observations are indicative of an inter-relationship between voltage-dependent and receptor-operated Ca^{2+} channels is, at present, unclear.

We have investigated Cch-induced Ca^{2+} mobilization in a salivary epithelial cell line, HSG-PA, derived from a human submandibular gland. We report here evidence for two modes of muscarinic receptor-operated Ca^{2+} entry in these cells, one which is associated with low agonist (Cch) concentrations ($K_{0.5} \leq 2.5$ μM) and another which is associated with relatively high concentrations of Cch ($K_{0.5} \sim 30$ μM). Only the latter component of Ca^{2+} entry appears to be associated with Ca^{2+} release. Ca^{2+} entry induced by low Cch concentrations is profoundly inhibited by membrane depolarization (high KCl, gramicidin D), whereas that induced by high Cch shows little if any inhibition. The Ca^{2+} response of these cells to Oxo-M, a muscarinic agonist which reportedly binds more selectively to high-affinity muscarinic receptors [35], is also markedly inhibited by membrane depolarization. These data are consistent with the hypothesis that there are two receptor-operated Ca^{2+} entry pathways in the HSG-PA cells, one activated by a low-affinity muscarinic receptor which also activates intracellular Ca^{2+} release and another activated by a high-affinity receptor which induces little if any Ca^{2+} release. This latter pathway may exhibit voltage-dependent gating.

Materials and Methods

Materials

Quin-2/AM and DTPA were purchased from Calbiochem. Fura-2/AM was from Molecular Probes. The following compounds were from Sigma: EGTA, HEPES, BSA, Cch, gramicidin D, verapamil, diltiazem and nifedipine. Myo[2-^{3}H]inositol (20 Ci/mmol) was purchased from Amersham and AG 1-×8 was from Bio-Rad.

Cell Culture

Experiments were performed on a human submandibular duct cell line, HSG-PA, a kind gift from Dr. Mitsunobu Sato [31]. HSG-PA cells were cultured at 37°C in a humidified 5% CO_2 atmosphere in EMEM supplemented with 10% newborn calf serum, 100 U ml^{-1} penicillin G and 100 μg ml^{-1} streptomycin sulfate (all from Biofluids). Cells were subcultured twice weekly. HSG-PA cells from passages 9 to 30 were used in these studies. In some experiments with fura-2, HSG-PA cells grown in a mixture of 50% Dulbecco's MEM and 50% Ham's F12 medium supplemented as described above, were employed. Results from these cells were indistinguishable from those obtained with cells grown in EMEM.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured using the fluorescent dye quin-2 essentially as previously described [11]. Briefly, cells were grown to confluence and detached by treatment with Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution containing 4 mM EGTA and 10 mM HEPES, pH 7.4, for about 7 min at 37°C. The cells were collected by brief centrifugation (15 sec) and resuspended in BSS (NaCl 130 mM; KCl 5 mM; $MgCl_2$ 1.0 mM; $CaCl_2$ 1.5 mM; glucose 10 mM; HEPES 20 mM buffered to pH 7.4 with Tris base). The cell suspensions (~6×10^6 cells ml^{-1}) were preincubated at 37°C for 5 min and then were incubated with 10 μM quin-2/AM for a further 20 min. Thereafter, the cells were washed twice and resuspended in BSS containing 1 mg ml^{-1} BSA and kept at room temperature. Just before fluorescence measurements were performed, the cells were again centrifuged and resuspended (~2×10^6 cells ml^{-1}) in the same medium except where indicated. Fluorescence was measured at 37°C in a SLM-8000 microprocessor-controlled spectrofluorimeter as previously described [13]. Fluorescence due to external quin-2 was determined in each experiment using a 50 nM Mn^{2+} and 100 nM DTPA quench protocol [28]. Calibration of the fluorescence signal and calculation of $[Ca^{2+}]_i$ were as described by Tsien, Pozzan and Rink [32].

Cells were also prepared for $[Ca^{2+}]_i$ measurements with fura-2 as described above for quin-2 except that loading with the dye was carried out by incubation with 1-μM fura-2/AM for 20 min at 37°C. Fura-2 fluorescence measurements were performed and analyzed as described by Grynkiewicz, Poenie and Tsien [10] using excitation wavelengths of 340 and 380 nm and measuring emission at 510 nm.

Analysis of Inositol Phosphates

Confluent cells were labeled with [^{3}H]myoinositol (4 μCi ml^{-1}; 12 ml/100-mm dish) in growth medium for 40 hr. Labeled cells were then detached and loaded with quin-2/AM as described above. Just before measurement of inositol phosphates, the cells were centrifuged and resuspended (~2×10^6 cells ml^{-1}) in a Ca^{2+}-free medium (BSS without Ca^{2+}, containing BSA). Cells (1.5 ml) were then incubated at 37°C with various agents for 20 sec. Incubations were terminated by adding 250 μl of 100% trichloroacetic acid, after which the cells were vortexed and kept on ice for at

least 10 min, and then centrifuged for 15 sec. The supernatant was removed, extracted four times with 5 volumes of ethyl ether, neutralized with 0.1 N NaOH and placed on a 1-ml ion-exchange column (AG 1-×8, 200–400 mesh, formate form). Different [^{3}H]inositol phosphates were eluted from the column as described previously [3]. The radioactivity in each 5-ml eluate was determined by liquid scintillation spectrometry and the results were expressed as cpm/3 × 10^6 cells. All data are presented as mean ± SE and were tested for statistical significance by Student's *t* test.

Results

Effect of Cch on Ca^{2+} Release and Ca^{2+} Entry

Figure 1 shows typical Cch-induced changes in $[Ca^{2+}]_i$ in HSG-PA cells. For cells in a complete Ca^{2+}-containing medium, stimulation with 100 μM Cch resulted in a rapid rise in $[Ca^{2+}]_i$ from 121 ± 10 nM to 560 ± 59 nM (n = 15) within 20 sec. $[Ca^{2+}]_i$ then declined to resting levels within about 5 min (Fig. 1*a*). This response was fully inhibited by pretreatment of cells with 10 μM atropine (*not shown;* [11]). Cch-induced Ca^{2+} mobilization was further examined using a previously established approach [12] in which cells were first stimulated with Cch in a nominally Ca^{2+}-free medium to observe intracellular Ca^{2+} release; thereafter, Ca^{2+} was re-added to the incubation medium to observe extracellular Ca^{2+} entry (Fig. 1*b,c*). After Cch (100 μM) stimulation in a Ca^{2+}-free medium, $[Ca^{2+}]_i$ rapidly increased from 104 ± 11 nM to 329 ± 38 nM (n = 15), declining to a value close to initial levels within 2.5 min (Fig. 1*b*). This response is believed to represent Ca^{2+} release from intracellular Ca^{2+} stores. A second rise of $[Ca^{2+}]_i$ (from 84 ± 6 nM to 244 ± 17 nM; n = 15) was evoked within 40 sec of Ca^{2+} reintroduction into the medium (final $[Ca^{2+}]$, 1.5 mM, typically added 3 min after stimulation with Cch, Fig. 1*b*). This second $[Ca^{2+}]_i$ rise declined relatively slowly, as in Fig. 1*a*, returning to resting levels after about 5 min, and is considered to represent Ca^{2+} entry across the plasma membrane. In the absence of Cch, the addition of Ca^{2+} to this nominally Ca^{2+}-free medium results in no alteration in $[Ca^{2+}]_i$ from resting levels (*not shown*). Thus, at 100 μM Cch, both the peak Ca^{2+} release and Ca^{2+} entry responses are ~3 times basal values (*see also* ref. 12). We have previously shown that atropine completely blocks both of the responses shown in Fig. 1*b* [11].

When cells were stimulated in a Ca^{2+}-free medium with a lower Cch concentration (e.g., 2.5 μM. Fig. 1*c*), the first response (Ca^{2+} release) appeared relatively more diminished (average peak increase in $[Ca^{2+}]_i$, 24 ± 11 nM, n = 7) than the second (Ca^{2+} entry; average peak increase in $[Ca^{2+}]_i$, 39 ± 10 nM, n = 7). This observation is illustrated more quantitatively in Fig. 2. Here we analyzed the effect of Cch concentration on peak $[Ca^{2+}]_i$ due to Ca^{2+} release and Ca^{2+} entry using a Scatchard plot. The points corresponding to Ca^{2+} release fall on a good straight line (Fig. 2*a*), consistent with the existence of a single relatively low-affinity Cch site ($K_{0.5} \cong 30$ μM, *see* figure caption) associated with this phenomenon. The Scatchard plot of the Ca^{2+} entry data, on the other hand, is clearly curvilinear indicating the existence of (at least) two Cch-associated components (Fig. 2*b*). These Ca^{2+} entry data are consistent with a two-site model; a low-affinity site with $K_{0.5}$ close to that of the Cch site associated with Ca^{2+} release, and a high-affinity site with $K_{0.5} \leq 2.5$ μM (*see* figure caption).

Figure 2*c* shows the Scatchard analysis of Ca^{2+} release data obtained in a similar fashion to Fig. 2*a*

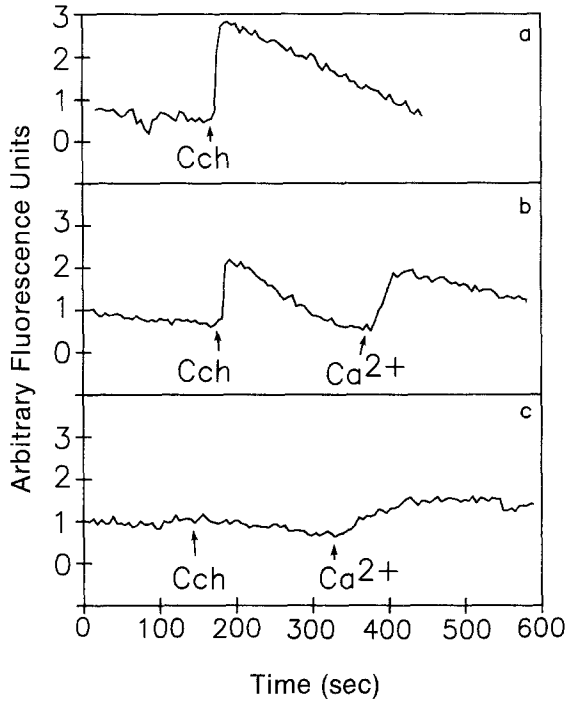

Fig. 1. The effect of Cch on $[Ca^{2+}]_i$ transients in HSG-PA cells. $[Ca^{2+}]_i$ was measured by quin-2 fluorescence as described in Materials and Methods. Cells were preincubated in a complete medium (*a*) or a Ca^{2+}-free medium (*b,c*) for 5 min at 37°C, and then Cch (*a,b*, 100 μM; *c*, 2.5 μM) and Ca^{2+} (1.5 mM) were added as indicated by the arrows. The traces shown here are typical of 15 (*a,b*) or six (*c*) experiments performed with different cell preparations. Similar results to those shown in *b* are obtained with this protocol when cells are incubated in a Ca^{2+}-free medium to which 0.5 mM EGTA has been added [12]. *See* text for quantitation of $[Ca^{2+}]_i$

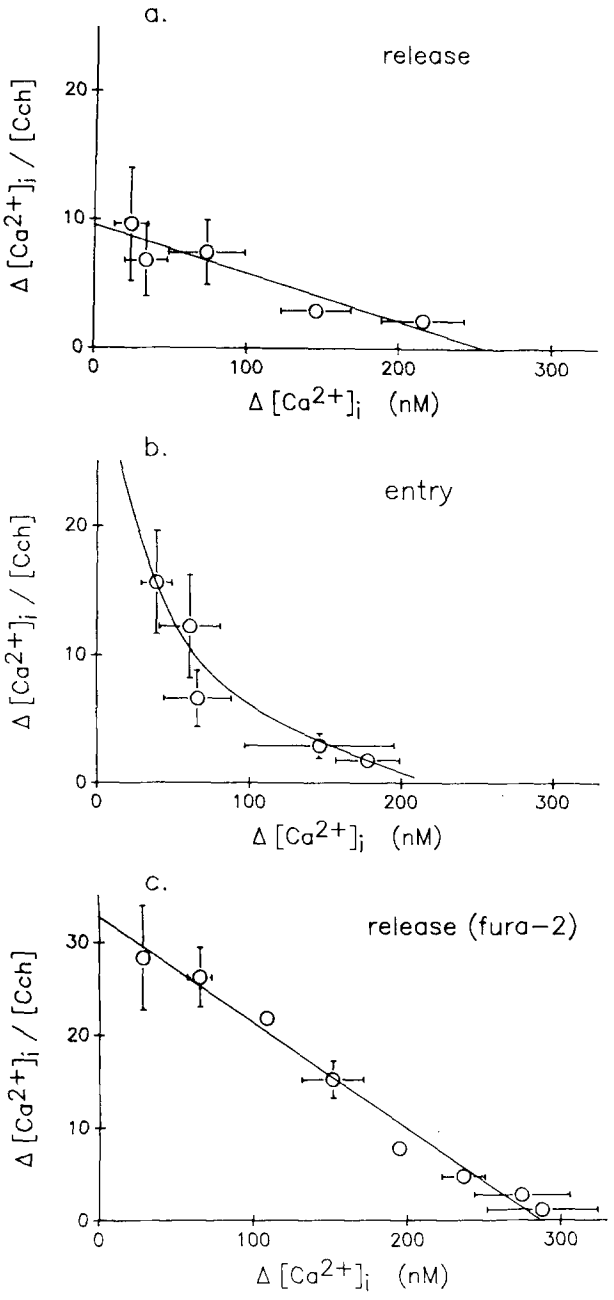

Fig. 2. Effect of different concentrations of Cch on Ca^{2+} release and Ca^{2+} entry in HSG-PA cells. The data are illustrated as Scatchard plots [30]. (*a,b*) Cells were incubated and stimulated with Cch as in Fig. 1*b,c*, using quin-2 to measure the Ca^{2+} responses. The peak values of the first Ca^{2+} response (Ca^{2+} release), determined 20 sec after stimulation with Cch, are shown in *a*. The peak values of the second Ca^{2+} response (Ca^{2+} entry) determined 40 sec after re-addition of Ca^{2+} are shown in *b*. (*c*) Shows the peak values of Ca^{2+} release measured with fura-2 in complete BSS containing in addition 1.64 mM EGTA. The lines drawn through the data points in *a* and *c* were obtained by linear least-squares regression and give $K_{0.5}$ values of 27 ± 4 μM and 8.8 ± 0.2 μM, respectively, for the effect of Cch. The line drawn through the data points in *b* corresponds to a two-site model with $K_{0.5}$ values for Cch of 1.5 and 30 μM. This line was fit by trial and error. Data points shown here are the mean ± SE of at least five separate experiments (*a,b*) or three separate experiments (*c*) performed with different cell preparations

but using the more sensitive Ca^{2+} indicator fura-2 [10]. These experiments were carried out in order to test the possibility that a high-affinity Cch site associated with Ca^{2+} release was overlooked in Fig. 2*a* owing to possible $[Ca^{2+}]_i$ buffering by quin-2. These data show comparable maximal levels of Ca^{2+} release (i.e., similar intercepts on the horizontal axis of the Scatchard plot) but higher levels of release at low [Cch]. This result is consistent with the hypothesis that higher levels of Ca^{2+} release are observed with fura-2 owing to the lower levels of indicator used and the concomitant reduction in buffering of $[Ca^{2+}]_i$. However, the Scatchard plot of these data is nevertheless linear indicating that a second Cch site associated with Ca^{2+} release is not unmasked by this maneuver and thus providing strong evidence that the high-affinity Cch site associated with Ca^{2+} entry in Fig. 2*b* is not associated with Ca^{2+} release. Owing to the rapid loss of fura-2 by the HSG-PA cells at 37°C (*not shown*), we have employed the less permeant dye quin-2 in the subsequent experiments reported here.

Effects of Membrane Depolarization on Ca^{2+} Release and Ca^{2+} Entry

When HSG-PA cells were incubated in a high K^+ medium (>55 mM), no significant effects were observed on the two phases of Ca^{2+} mobilization induced by 100 μM Cch (release, from 98 ± 17 nM to 329 ± 92 nM, and entry from 83 ± 11 nM to 216 ± 43 nM; $n = 7$) except for a slight delay in reaching peak values for the latter response (approximately 1 min after addition of Ca^{2+} in high K^+ medium, Fig. 3*a*, *vs.* approximately 40 sec in BSS, Fig. 1*b*). However, the Ca^{2+} entry response induced by 2.5 μM Cch was completely inhibited by high K^+ (Fig. 3*b* compared with Fig. 1*c*). This effect of high K^+ on Ca^{2+} entry appeared to be due to membrane depolarization since gramicidin D (1 μM) had a similar effect (*not shown*). These data further support the contention that the component of Ca^{2+} entry observed at low [Cch] in Fig. 2*b* is mediated by a different process than that observed at high [Cch].

Oxo-M Induced Ca^{2+} Mobilization in HSG-PA Cells

To further investigate the possibility that the Ca^{2+} entry observed at low [Cch] is related to a high-affinity muscarinic receptor we investigated the effects of Oxo-M, a muscarinic agonist believed to selectively bind to high-affinity muscarinic receptor sites [35]. As shown in Fig. 4*a*, Oxo-M (100 μM) evoked a rapid increase in $[Ca^{2+}]_i$, peaking at about 30–40 sec and declining back to initial levels within 5 min. The average increase in Ca^{2+} was from 72 ±

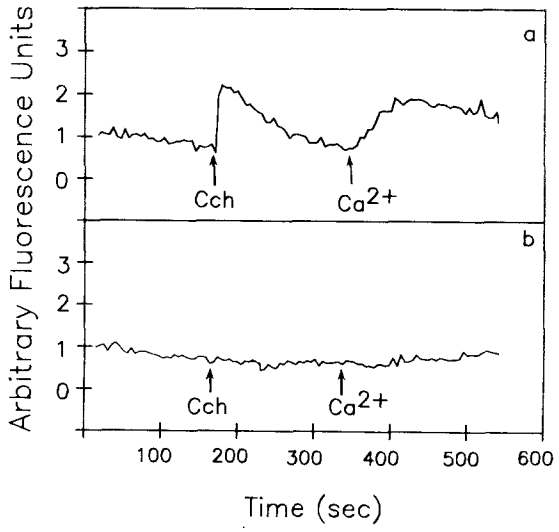

Fig. 3. Effect of membrane depolarization on Cch-induced Ca^{2+} release and Ca^{2+} entry in HSG-PA cells. Quin-2 fluorescence transients were measured exactly as in Fig. 1*b,c* except cells were incubated in a high K^+ medium (135 mM). Cch (*a*, 100 μM; *b*, 2.5 μM) and Ca^{2+} (1.5 mM) were added as shown by the arrows. The traces shown here are typical of experiments performed with at least three different cell preparations. *See* text for quantitation of $[Ca^{2+}]_i$

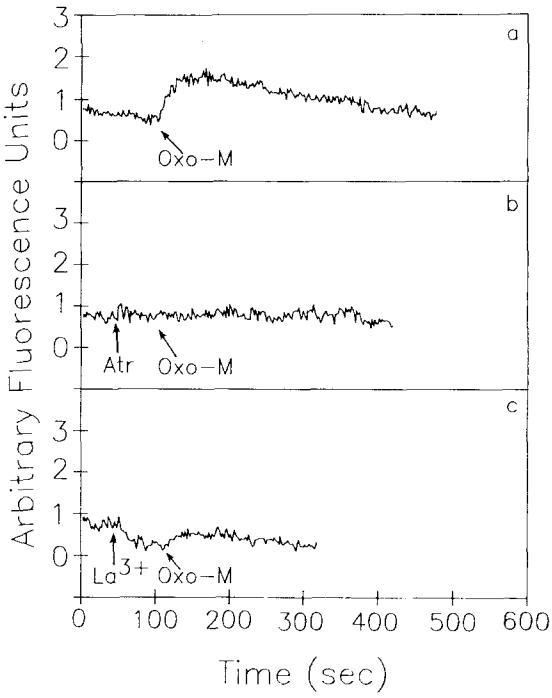

Fig. 4. Effect of Oxo-M on $[Ca^{2+}]_i$ in HSG-PA cells. Confluent cells were loaded with 10 μM quin-2/AM, incubated in a complete medium, and $[Ca^{2+}]_i$ was measured as described under Materials and Methods. Oxo-M (100 μM); atropine (Atr, 10 μM), and La^{3+} (50 μM) were added at the arrows. The traces shown here are typical of experiments performed with at least three cell preparations. *See* text for quantitation of $[Ca^{2+}]_i$

2 nM to 104 ± 5 nM, $P < 0.001$, $n = 8$. This response was completely inhibited by atropine (Fig. 4*b*) and appeared to result mainly from Ca^{2+} entry across the plasma membrane since it was profoundly blocked (~75%) by 25–50 μM La^{3+} (Fig. 4*c*; average increase in $[Ca^{2+}]$ after La^{3+} was from 46 ± 8 nM to 54 ± 9 nM, $n = 3$). As previously shown by us, La^{3+} potently blocks Ca^{2+} entry in HSG-PA cells and is without effect on intracellular Ca^{2+} signals monitored with quin-2 in these cells (*see* Fig. 3*b* and Table 2 in ref. 12). In addition, the rise in $[Ca^{2+}]_i$ induced by Oxo-M was substantially inhibited by preincubating the cells in either a high K^+ medium (~90%; Fig. 5*a*, average increase in $[Ca^{2+}]_i$, 4 ± 5 nM, $n = 5$) or a physiologic medium containing gramicidin D (~70%; Fig. 5*b*, average increase in $[Ca^{2+}]_i$, 10 ± 4 nM). However, the organic Ca^{2+} channel blockers verapamil (10 μM), diltiazem (10 μM) and nifedipine (1 μM) appeared to have no significant effects on the Oxo-M response (Fig. 5*c–e*). The entry of Ca^{2+} induced by both low (2.5 μM) and high (100 μM) concentrations of Cch was similarly unaffected by these agents ([12]; *data not shown*).

The Effect of Cch and Oxo-M on IP_3 Generation

We next examined the ability of Cch and Oxo-M to induce the formation of IP_3 in HSG-PA cells. As shown in the Table, 100 μM Cch increases IP_3 production by ~55% within 20 sec. A low concentration (1 μM) of Cch resulted in a smaller increase in IP_3 above basal levels (~25%). Similar results were obtained with 100 μM Oxo-M (~20% increase). It is generally accepted that intracellular Ca^{2+} release from intracellular stores is the result of the second messenger IP_3 acting at its receptor on these stores [2]. It has been suggested, but not unequivocally

Table. $[^3H]IP_3$ generation due to Cch and Oxo-M in HSG-PA cells[a]

Experimental conditions	$[^3H]IP_3$ generation (% control)
Control	100
100 μM Cch	[b]154 ± 19
1 μM Cch	[b]126 ± 9
100 μM Oxo-M	[c]120 ± 14

[a] Cells were incubated with buffer (control) or with the concentrations of Cch or Oxo-M indicated for 20 sec at 37°C. Thereafter incubations were terminated and analyzed for $[^3H]IP_3$ generation as described in Materials and Methods. The data shown here are the mean ± SE from at least three separate experiments. Control IP_3 production = 3958 ± 366 cpm/3 × 10^6 cells, $n = 6$.
[b] Significantly different from control, $P < 0.05$.
[c] Not significantly different from control.

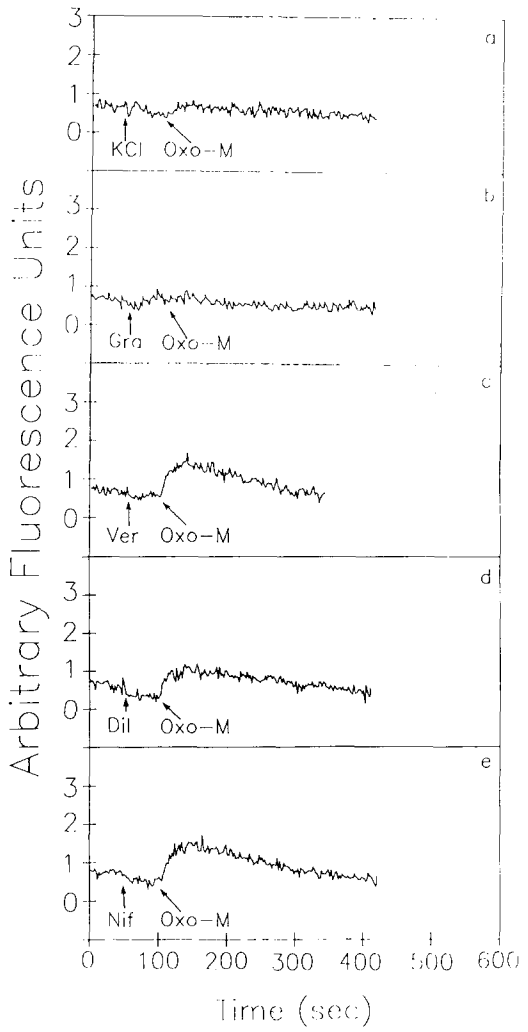

Fig. 5. Effect of membrane depolarization and Ca^{2+} channel blockers on Oxo-M induced Ca^{2+} mobilization in HSG-PA cells. $[Ca^{2+}]_i$ was measured as in Fig. 4. Oxo-M (100 μM), KCl (55 mM), gramicidin D (Gra, 1 μM), verapamil (Ver, 10 μM), diltiazem (Dil, 10 μM), and nifedipine (Nif, 1 μM) were added at the arrows. The traces shown are typical of experiments performed with at least three cell preparations. *See* text for quantitation of $[Ca^{2+}]_i$

proven, that receptor-mediated Ca^{2+} entry is mediated by IP_3 and its metabolite IP_4 [22]. The smaller changes in IP_3 production are consistent both with the lower Ca^{2+} release response seen in HSG-PA cells with 2.5 μM Cch (Fig. 2) and with the apparently small Ca^{2+} release component observed with 100 μM Oxo-M (Fig. 4*c*).

Discussion

The data presented in this report are consistent with an hypothesis suggesting that in the human salivary epithelial cell line, HSG-PA, there exist two modes by which a muscarinic agonist may stimulate Ca^{2+} entry. One, seen only at higher concentrations of Cch, is associated with a marked increase in IP_3 formation and pronounced release of Ca^{2+} from intracellular stores, and is unaffected by membrane depolarization. The other, seen at low concentrations of Cch or with Oxo-M use, is associated with smaller increases in IP_3 formation and little release of Ca^{2+} from intracellular stores, and is blocked by membrane depolarization.

A dissociation between Ca^{2+} release and entry was previously observed by us in HSG-PA cells treated with an active phorbol ester [11], by Valone and Johnson in platelets [33], and by Owen in murine lymphocytes [25]. Similarly, others have used low concentrations of agonists to induce Ca^{2+} entry without detectable inositol phosphate generation or Ca^{2+} release (for review, *see* ref. 7). Furthermore, some recent experiments, using rapid time resolution or Mn^{2+} quench procedures, indicate that Ca^{2+} entry can occur before Ca^{2+} release [5, 18, 19, 29]. All of these observations are consistent with the suggestion that there are two distinct receptor-operated Ca^{2+} entry mechanisms; one associated with the receptor-induced Ca^{2+} release response (and likely related to IP_3 generation) and the other dissociated from this response.

As mentioned earlier, there may exist some overlap between the properties of classical voltage-gated Ca^{2+} channels and receptor-operated Ca^{2+} channels [1, 6, 8, 9, 14, 16, 17, 20, 24, 27, 34, 36]. However, a direct relationship between membrane potential gating and receptor-operated Ca^{2+} entry has not been clearly established. There are a few reports, also cited earlier, which suggest that Ca^{2+} influx via receptor-operated pathways is modulated by membrane potential in some systems [8, 9, 16, 20, 24, 34]. It is possible that the reduction in Ca^{2+} entry due to membrane depolarization observed in some of these systems can be simply explained as the result of a reduced electrochemical gradient for Ca^{2+} which is driving Ca^{2+} entry via an electrogenic Ca^{2+} channel. However, it seems unlikely that this effect can account for the profound inhibition of Ca^{2+} entry observed here at low Cch concentrations or with Oxo-M. The contribution of the chemical gradient for Ca^{2+} to the electrochemical potential difference of this cation in HSG-PA cells is $= (RT/F)\ln([Ca^{2+}]_o/[Ca^{2+}]_i) \approx 260$ mV) (assuming $[Ca^{2+}]_i = 100$ nM). Thus a membrane potential of -50 mV can only account for ~30% of the total driving force for electrogenic Ca^{2+} entry, and even a complete depolarization of the membrane (which is unlikely under the experimental conditions employed here) would be expected to yield substantial residual Ca^{2+} influx. Although considerable further experimental work is obviously required on this

point, these data suggest that the Ca^{2+} entry pathway associated with low Cch concentrations or use of Oxo-M in HSG-PA cells may be voltage gated. This does not, however, appear to be a classical voltage-dependent Ca^{2+} channel since it is apparently closed rather than opened by membrane depolarization, and since it is not affected by the organic channel blockers verapamil, diltiazem and nifedipine (although recently voltage-dependent channels which are insensitive to these agents have been identified, *see* ref. 21).

At least four subtypes of muscarinic receptors are known to exist and it has been suggested that each subtype may couple to an unique type of effector system leading to various biochemical and physiological responses [15]. Since Oxo-M selectively binds to high-affinity receptors [23, 35], it would seem reasonable to hypothesize, based on our results, that the low-affinity type of muscarinic receptor may induce Ca^{2+} entry via a mechanism associated with intracellular Ca^{2+} release, while the high-affinity type of muscarinic receptor may induce Ca^{2+} entry via a mechanism dissociated from intracellular Ca^{2+} release.

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