Muscarinic Signaling Pathway in Submandibular Cells of Adult and Early Postnatal Rats (43628)

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Abstract. **Elements of the muscarinic signal transduction pathway were compared in submandibular acinar cells of 1-day-old, 1-week-old, and adult rats after exposure to concentrations of acetylcholine ranging from 0.05 to 10 μM. Formation of inositol trisphosphate (IP_3) and intracellular Ca^{2+} were comparable in cells from the three age groups after exposure to agonist concentrations <1 μM. At higher agonist concentrations, IP_3 generation and peak initial changes in $[Ca^{2+}]_i$ were significantly greater in cells of newborn animals. In cells of 1-week-old animals, increased peak $[Ca^{2+}]_i$ responses were seen even at low agonist concentrations, although IP_3 production was not increased when compared with fully mature cells. Increased initial $[Ca^{2+}]_i$ peaks, but comparable subsequent plateau $[Ca^{2+}]_i$ values, were seen in the immature cells in both Ca^{2+}-containing and Ca^{2+}-free solutions. Permeabilized cells of early postnatal animals took up less $^{45}Ca^{2+}$ into nonmitochondrial Ca^{2+} pools in the presence of 1.5 mM ATP and also released less tracer in response to intermediate IP_3 concentrations than adult cells. Developing salivary cells thus show differences in important functional linkages of the muscarinic signal transduction pathway, including those between receptor activation and phosphoinositide turnover and between IP_3 and release of internally stored Ca^{2+}. Differences in the Ca^{2+} stores or in their sensitivity to IP_3 may account for the latter observation. Immature salivary cells seem to have adequate mechanisms for Ca^{2+} entry.** [P.S.E.B.M. 1993, Vol 203]

The major salivary glands of rodents undergo considerable postnatal development, from a relatively undifferentiated state at birth to fully mature organs by 12 weeks of age (1–4). Morphologic differentiation is associated with the parallel development of the mechanisms responsible for the formation of saliva, particularly of the ion transport systems associated with the secretion of water and electrolytes (5–9). Saliva secretion is deficient in the early neonatal period and is markedly impaired until 4 weeks of age (10). The decreased ability to secrete saliva correlates with smaller fluxes of K^+ (6, 9) and Cl^- (9, 11, 12), which are essential components of the fluid secretory mechanism in acinar cells (13). Efflux of these ions upon stimulation occurs by way of Ca^{2+}-regulated membrane channels (14), but salivary cells of newborn rats had a normal or slightly enhanced Ca^{2+} response when exposed to a single optimal dose of acetylcholine (11). The increase in intracellular Ca^{2+} that occurs upon stimulation is part of a functional cascade or signal transduction mechanism that includes activation of phospholipase C, increased turnover of membrane phosphoinositides, formation of inositol trisphosphate (IP_3) and other metabolites, the release of Ca^{2+} from intracellular storage sites, and the influx of Ca^{2+} from the extracellular space (14). In addition to the apparent uncoupling between the Ca^{2+} response and the efflux of K^+ and Cl^- already documented (9, 11–13), immature salivary cells may demonstrate functional dissociations in other aspects of the signaling pathway, which may help explain the reduced ability to secrete saliva. Of particular interest in this context is their ability to produce IP_3 and the relative contributions of internal Ca^{2+} release and of Ca^{2+} influx to overall Ca^{2+} mobilization. In this study, therefore, we investigated these

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functional relationships in mature and immature submandibular cells by comparing dose-related responses to acetylcholine in terms of IP_3 formation and of changes in intracellular Ca^{2+} measured with a Ca^{2+} fluorescent probe. We also compared the ATP-dependent $^{45}Ca^{2+}$ uptake and the release of this tracer from nonmitochondrial pools of cells permeabilized with detergent upon exposure to graded concentrations of 1,4,5-IP_3.

Materials and Methods

Cell Preparation. Cell aggregates were prepared from the submandibular glands of 1-day-old, 1-week-old, and adult rats as described previously (11). Sprague-Dawley rats were anesthetized with Nembutal and euthanized by exsanguination just before removal of the submandibular gland. Cell clusters were prepared by digestion of gland fragments with hyaluronidase and purified collagenase in an oxygenated, Hepes-buffered, Krebs-Ringer medium of the composition described previously (11), followed by trituration through graded plastic pipettes. The resulting suspensions were then washed three times in medium containing 2% bovine serum albumin, with an intermediate filtering through nylon mesh, before resuspension in a similar medium containing 1 m*M* $CaCl_2$. Each preparation for fura-2 studies consisted of glands from one adult rat, 9–11 1-week-old rats, or 20–25 1-day-old rats, to provide similar quantities of tissue. For the IP_3 assays and the permeabilized cell experiments, the numbers were doubled. In a few cases, samples from a preparation that was to be used primarily for IP_3 measurements or from cells destined for permeabilization were also used for the measurement of Ca^{2+} responses with the fura-2 method. All data reported represent the results of at least three experiments on separate cell preparations.

Measurement of Inositol Triphosphate Formation. Measurement of inositol trisphosphate formation was performed by a competitive radioreceptor binding assay (15, 16), as modified by Deanin *et al.* (17). Cells were incubated with the desired agonist concentration and cell extracts were prepared at Time 0 (before agonist addition), and after 15 sec, 30 sec, and 1 min. The reactions were terminated by adding an equal volume of 16% trichloroacetic acid and incubating on ice for 15 min. The protein pellets were removed by centrifugation in an Eppendorf microfuge and the supernatant was extracted five times with 3 vol of water-saturated ether. The resulting extracts were neutralized with 10 m*M* $NaHCO_3$ with 5 m*M* EDTA (final concentrations). Crude IP_3 receptor-containing membranes, prepared from bovine cerebelli (16), were incubated with [^{3}H]inositol-1,4,5-trisphosphate in the presence of the cell extracts, or known concentrations of pure inositol-1,4,5-trisphosphate in trichloroacetic acid/ether-extracted buffer to create a standard curve. Results were normalized to the protein content of the individual samples (assayed in the protein pellet after incubation overnight in 0.15 *M* NaOH).

Measurements of Intracellular Ca^{2+} with Fura-2. Intracellular calcium was measured using ratiometric fluorometry of the Ca^{2+}-sensitive fluorescent indicator fura-2 as described previously (12) using a PTI Deltascan fluorometer. Cells were loaded with 1 μM fura-2 as the acetoxymethyl ester for 30 min at 37°C, followed by resuspension for 30 min in fresh medium, to permit completion of hydrolysis of the ester. The suspensions were top-gassed with O_2 every 10 min throughout this procedure. Cells were then placed at room temperature under a constant stream of O_2. Cells treated identically except for fura-2 loading served to correct for background fluorescence. Aliquots (2 ml) of the fura-2-loaded suspensions were resuspended in fresh Ca^{2+}-containing medium immediately before assay, placed in 1-cm quartz cuvettes, and excited with alternating 340- and 380-nm light. Acetylcholine was added through an injection port to final concentrations ranging from 0.05 to 10 μM. In other experiments, cells were resuspended in a buffer containing 0.1 m*M* EGTA and no added $CaCl_2$ immediately before placing the cuvette in the instrument for the fluorescence measurements. In the latter experiments, 1 m*M* $CaCl_2$ was added at the end of the run. Finally, for each assay, calibration was performed by sequential addition of 75 μM ionomycin, 0.0005% digitonin, and 10 m*M* EGTA. The ratio of the emissions at 505 nm for the two excitation wavelengths was then converted to $[Ca^{2+}]_i$ by the equations of Grynkiewicz *et al.* (18). The peak responses reported below represent the highest point in the trace (usually 3–5 sec after the addition of the agonist) and the plateau values are the average Ca^{2+} levels observed between 240 and 270 sec after agonist addition.

^{45}Ca Uptake and Release in Permeabilized Cells. In order to determine the ability of IP_3 to release Ca^{2+} from internal stores, experiments were performed on cells permeabilized to allow access of the non-cell-permeant IP_3 to the stores. Cell aggregates prepared as described above were incubated in a cytosolic-like buffer containing 20 m*M* NaCl, 100 m*M* KCl, 5 m*M* $MgCl_2$, 10 m*M* Hepes, 15 m*M* glucose, $CaCl_2$ buffered with EGTA to a final free Ca = 100 n*M* (confirmed with a Ca^{2+}-sensitive electrode), and 0.2% bovine serum albumin (pH 7.2). After 10 min in this buffer at 37°C, 45 μg/ml of saponin were added and the incubation was continued for 10 min. The cells were then pelleted by gentle centrifugation and resuspended in the same buffer (without saponin), containing an ATP-regenerating system of 6 m*M* phosphocreatine and 8 units/ml of creatinine phosphokinase, and the mitochondrial inhibitors oligomycin and antimycin (10 μM each). $^{45}Ca^{2+}$ (1 μCi/ml) was then added to the suspensions

and, 10 min later, ATP-dependent uptake was initiated by the addition of 1.5 m*M* ATP. After the $^{45}Ca^{2+}$ content had reached a steady state, efflux of the tracer was initiated with the addition of inositol-1,4,5-trisphosphate at concentrations ranging from 0.01 to 10 μM.

Protein. Proteins were assayed by the Lowry procedure (19), except for the pellets from the IP_3 assays, for which the Coomassie method was used, according to instructions provided by the supplier (Pierce, Rockford, IL).

Statistics. Data are presented as means and SE from several identical experiments (see Results for pertinent numbers). Significance was established using unpaired Student's *t* test (two-tailed).

Materials. Fura-2 was obtained from Molecular Probes, Eugene, OR. Collagenase was from Worthington, Freehold, NJ. ^{45}Ca and $[^3H]IP_3$ were from NEN/Dupont, Boston, MA. Ionomycin as the Ca^{2+} salt was obtained from Calbiochem, San Diego, CA. The Coomassie protein dye reagent was from Pierce. Other chemicals were from Sigma, St. Louis, MO.

Results

IP_3 Production. The production of IP_3 after stimulation with the various concentrations of acetylcholine was measured in cell aggregates derived from the three age groups. The results are shown in Figure 1. Cells from adult animals (Fig. 1A) showed a maximum IP_3 response of 31.9 pmol/mg protein when exposed to an agonist concentration of 5 μM. This was observed 15 sec after stimulation, with a subsequent decrease in the cells' IP_3 content between 15 and 60 sec (Fig. 1A). Lower doses of acetylcholine induced a smaller or no response in adult cells (Fig. 1A), and a higher dose produced a slightly (not significantly) smaller response. Cells of 1-day-old rats (Fig. 1C) had IP_3 responses similar to those of adult cells when exposed to the lower concentrations of agonist, but had a significantly larger response when exposed to higher (5–10 μM) doses of the agonist (Fig. 1C). Thus, the peak IP_3 formed after exposure to 5 μM acetylcholine was 55.7 pmol/mg protein (Fig. 1C). Cells derived from 1-week-old animals (Fig. 1B) had an even greater response at higher concentrations of the agonist, such that the peak IP_3

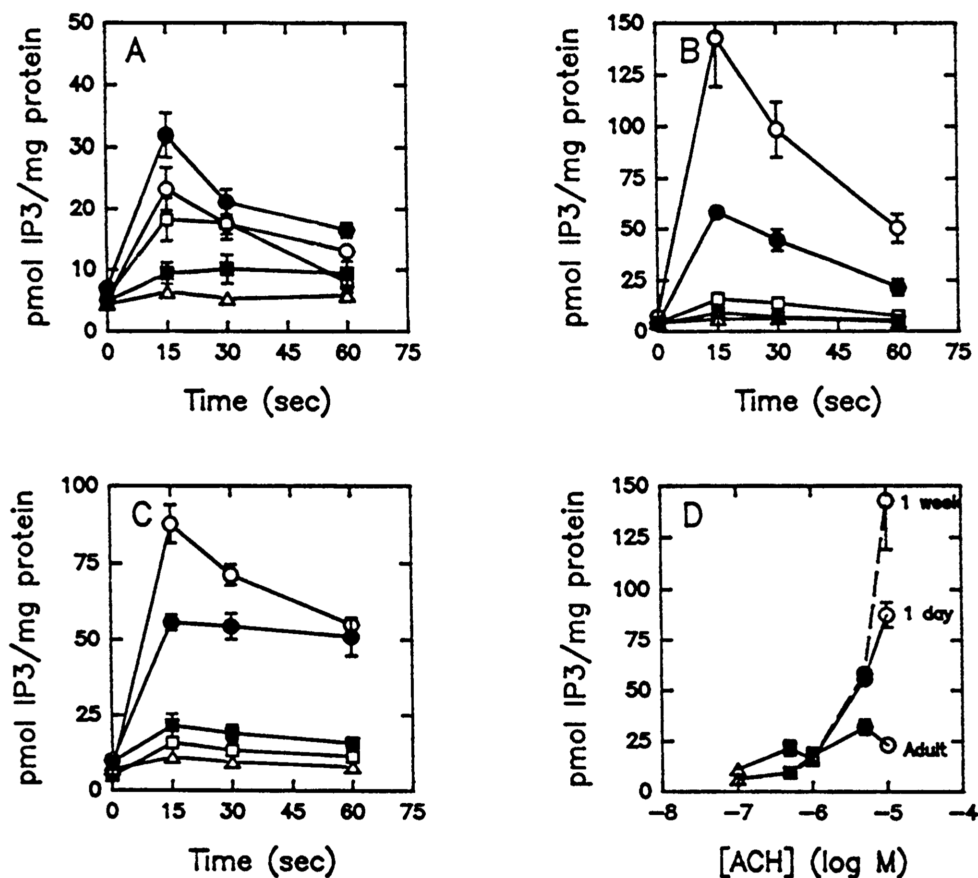

Figure 1. Inositol trisphosphate (IP_3) was determined as described in Materials and Methods after stimulation of submandibular cells with various concentrations of acetylcholine (open triangles, 0.1 μM; closed squares, 0.5 μM; open squares, 1 μM; closed circles 5 μM; and open circles, 10 μM). Data are shown for cells of adult (A), 1-week-old (B), or 1-day-old (C) animals. The peak response (15 sec) is replotted in 1D as a function of acetylcholine concentration. Results shown represent the mean and SE of five independent experiments under each condition.

Table I. Average Ca^{2+} Responses[a]

	$[Ca^{2+}]$ Ca^{2+}-containing buffer		$[Ca^{2+}]$ Ca^{2+}-free buffer		
	Peak (nM)	Plateau (nM)	Peak (nM)	Plateau (nM)	After Ca^{2+} addition (nM)
Adult					
[ACH]					
0	(88 ± 6)		(50 ± 3)		
5×10^{-8}	191 ± 29	186 ± 20	106 ± 7	41 ± 5	136 ± 3
10^{-7}	186 ± 17	169 ± 17	123 ± 26	42 ± 7	150 ± 3
5×10^{-7}	254 ± 39	128 ± 10	122 ± 24	37 ± 4	151 ± 18
10^{-6}	247 ± 32	154 ± 15	164 ± 46	35 ± 4	126 ± 32
5×10^{-6}	339 ± 36	184 ± 9	193 ± 40	62 ± 12	194 ± 35
10^{-5}	451 ± 56	198 ± 22	163 ± 33	43 ± 6	124 ± 24
1 Week old					
[ACH]					
0	(117 ± 6)		(63 ± 4)		
5×10^{-8}	227 ± 42*	162 ± 22	117 ± 21	48 ± 8	156 ± 23
10^{-7}	253 ± 4†	161 ± 2	192 ± 28	52 ± 7	194 ± 40
5×10^{-7}	566 ± 75†	179 ± 21	295 ± 63*	39 ± 10	204 ± 49
10^{-6}	748 ± 86‡	181 ± 22	304 ± 56	31 ± 3	227 ± 55
10^{-5}	945 ± 105†	168 ± 24	391 ± 34†	35 ± 6	217 ± 6*
1 Day old					
[ACH]					
0	(99 ± 5)		(53 ± 2)		
5×10^{-8}	135 ± 19	138 ± 14	89 ± 9	46 ± 7	149 ± 18
10^{-7}	178 ± 22	152 ± 14	85 ± 13	37 ± 8	118 ± 14
5×10^{-7}	236 ± 59	153 ± 16	134 ± 26	38 ± 7	149 ± 38
10^{-6}	375 ± 72	164 ± 13	173 ± 14	35 ± 8	163 ± 24
5×10^{-6}	708 ± 42†	161 ± 53	365 ± 44†	45 ± 2	203 ± 39
10^{-5}	724 ± 92*	174 ± 23	351 ± 52†	37 ± 6	166 ± 31

[a] Symbols (*, †, ‡) indicate significant difference at $P < 0.05$, 0.01, and 0.001, respectively, from values obtained in cells from adult animals under the same conditions ([acetylcholine; ACH] and presence or absence of Ca^{2+}).

formed was 58.4 pmol/mg protein at 5 μM acetylcholine and 142.7 pmol/mg protein at 10 μM agonist (Fig. 1B). Figure 1D shows the dose-response relationships for IP_3 formation at the earliest time point in the three populations of cells used in this study. It can be seen that IP_3 responses were similar at low doses of agonist, but at both 5 μM and 10 μM, the responses of the cells from the younger animals were significantly ($P < 0.01$) greater than those of the adults.

Changes in Intracellular Ca^{2+}. Changes in intracellular Ca^{2+} were investigated with fura-2 in cells from the three age groups during incubation in either Ca^{2+}-containing or Ca^{2+}-free solutions and after exposure to acetylcholine concentrations ranging from 0.01 to 10 μM. Tracings showing the average responses are shown in Figures 2–4 and the data are summarized in Table I. In the presence of external (medium) Ca^{2+}, cells from adult animals showed an initial peak increase in intracellular Ca^{2+} upon stimulation that became maximal at the highest agonist concentration (Fig. 2A). Resting Ca^{2+} (i.e., before stimulation) in these cells was 88 ± 6 nM and increased approximately 3-fold at 1 μM acetylcholine and 5-fold at 10 μM agonist (Fig. 2A). At the lower doses of agonist, the initial peak was approximately twice the resting levels (Table I). This initial peak was followed by a decline to a plateau value that ranged from 1.5 to 2.25 times the resting levels (Fig. 2A and Table I). In the absence of external Ca^{2+}, mature cells had lower resting and initial peak Ca^{2+} levels and even a 10-μM dose of acetylcholine only increased cell Ca^{2+} 2-fold (Fig. 2B and Table I). Similarly, the subsequent plateau was at a lower level than that seen in Ca^{2+}-containing solutions, and was not elevated above prestimulation level (Fig. 2B and Table I). On the addition of 1 mM Ca^{2+}, the plateaus increased to levels generally not significantly different from the plateau levels attained when the cells were stimulated in Ca^{2+}-containing buffer, and not significantly different among the different concentrations of acetylcholine.

The Ca^{2+} responses of cells isolated from 1-day-old rats in both Ca^{2+}-containing and Ca^{2+}-free incubation solutions are shown in Figure 3 and Table I. In the presence of external Ca^{2+}, the initial peak with 1 μM acetylcholine was nearly four times the resting levels, and with 10 μM agonist, the peak reached nearly seven times the resting levels (Fig. 3A and Table I). At these higher concentrations of agonist, a higher initial peak was observed than that seen in mature cells. At lower

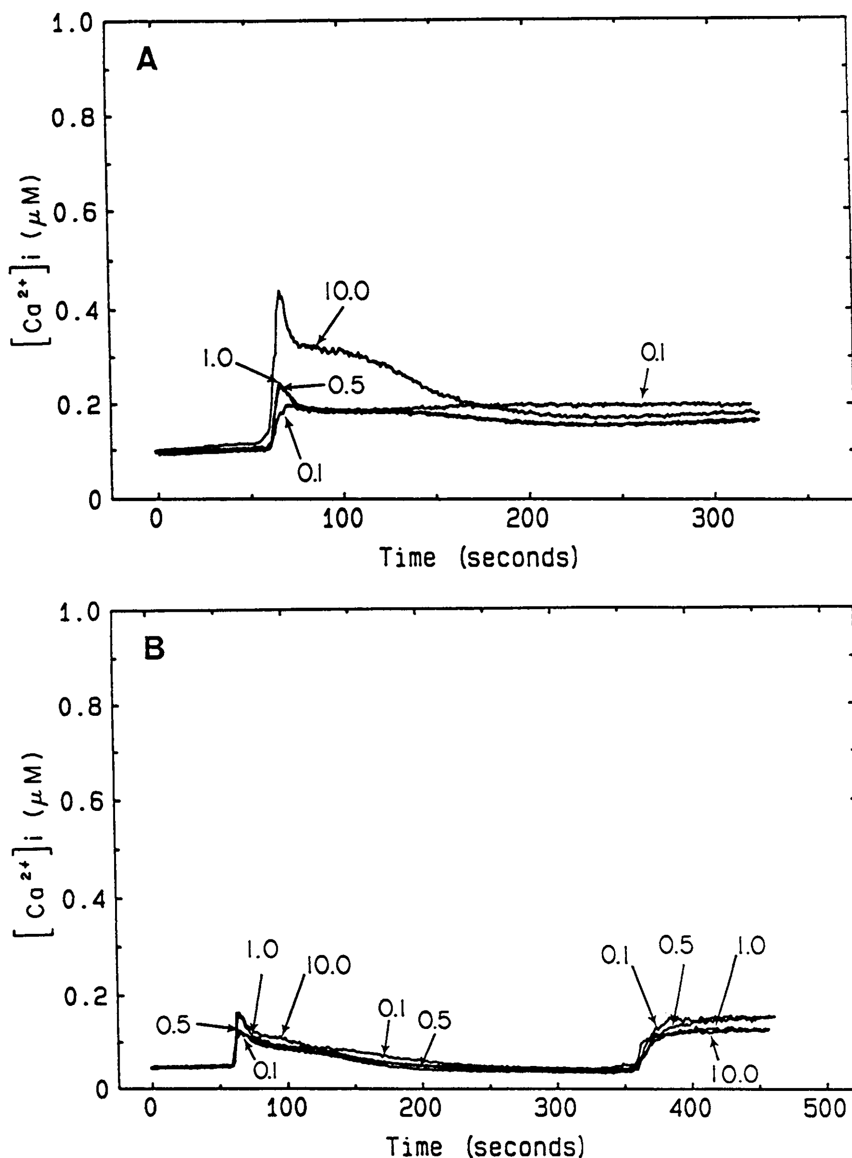

Figure 2. Intracellular Ca^{2+} in cells of adult animals was measured with fura-2 as described in Materials and Methods, in buffers containing 1 m*M* $CaCl_2$ (A) or 0.1 m*M* EGTA and no added $CaCl_2$ (B). Cells were exposed to the following concentrations of acetylcholine: 0.1 μM (curves marked 0.1), 0.5 μM (curves marked 0.5), 1.0 μM (curves marked 1), and 10 μM (curves marked 10). $CaCl_2$ (1 m*M*) was added to the curves in 2B at 360 sec. Curves represent the average results from five to eight separate experiments.

agonist concentrations (i.e., 0.05–0.5 μM), the initial Ca^{2+} peaks were similar to those seen in cells of adult animals (Fig. 3A and Table I). The subsequent plateau values of the Ca^{2+} were also comparable in adult and neonatal cells in Ca^{2+}-containing medium (Table I). Similar differences in the initial peak of the Ca^{2+} response were observed in Ca^{2+}-free solutions. At the higher concentrations of agonist, the initial peak attained was higher in cells isolated from newborn animals (compare Figs. 2B and 3B, Table I), but the plateau values were comparable to those of adults.

The Ca^{2+} responses of cells isolated from 1-week-old animals are shown in Figure 4 and Table I. The initial responses were also significantly higher in both types of incubation solution than those seen in cells of adult animals (Table 1). For example, the initial peak observed with 10 μM acetylcholine was more than eight times the resting levels in Ca^{2+}-containing solutions (Fig. 4A and Table I) and six times the resting levels in Ca^{2+}-free solutions (Fig. 4B and Table I). Plateau values, on the other hand, were comparable in either solution to those seen in cells of the other two age groups (Fig. 4 and Table I). Figure 5 shows the initial peak values of $[Ca^{2+}]_i$ at the various concentrations of agonist in Ca^{2+}-containing (Fig. 5A) and Ca^{2+}-free (Fig. 5B) buffers for the three cell populations studied and Figure 6 shows the plateau $[Ca^{2+}]_i$ in Ca^{2+}-containing (Fig. 6A) and Ca^{2+}-free buffer (Fig. 6B) in the three populations of cells that were used in these experiments. It can be seen that the initial peaks are clearly higher in

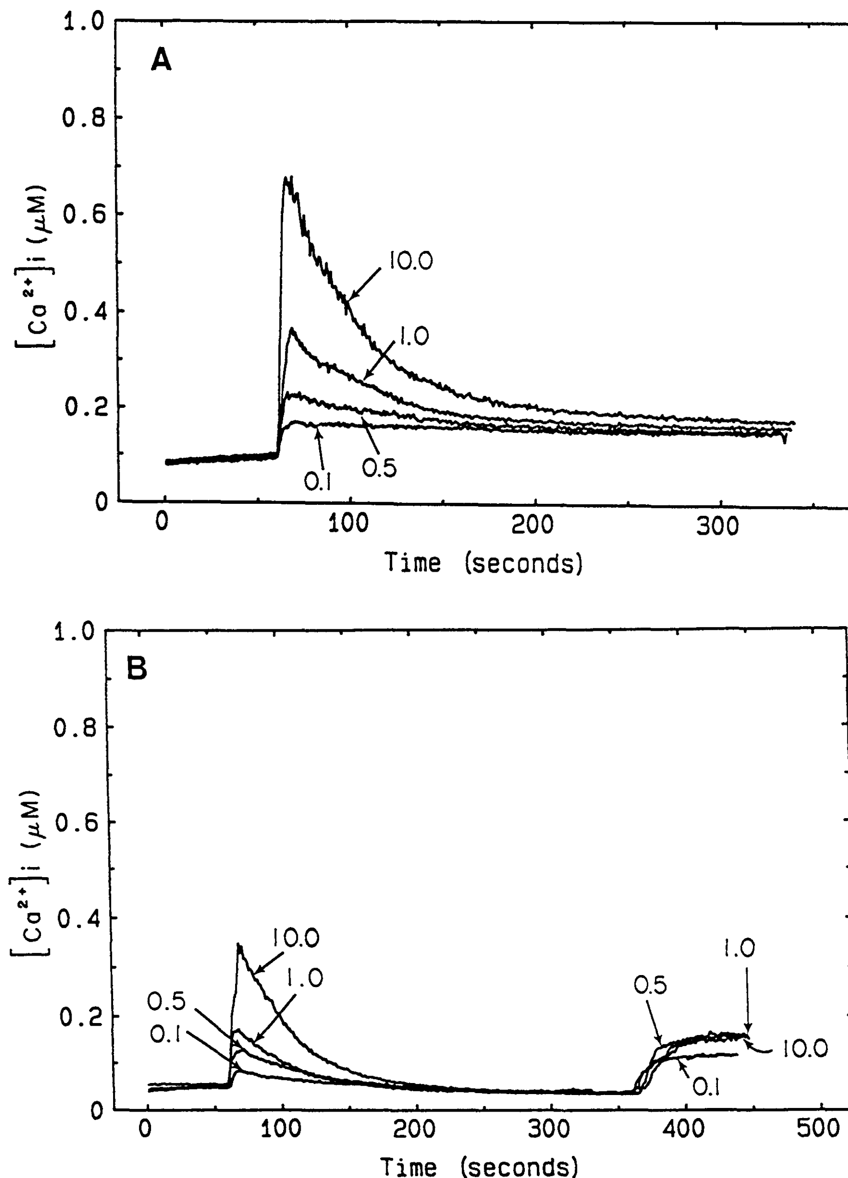

Figure 3. Intracellular Ca^{2+} in cells of 1-day-old animals was measured with fura-2 as described in Materials and Methods, in buffers containing 1 m*M* $CaCl_2$ (A) or 0.1 m*M* EGTA and no added $CaCl_2$ (B). Cells were exposed to the following concentrations of acetylcholine: 0.1 μ*M* (curves marked 0.1), 0.5 μ*M* (curves marked 0.5), 1.0 μ*M* (curves marked 1), and 10 μ*M* (curves marked 10). $CaCl_2$ (1 m*M*) was added to the curves in 3B at 360 sec. Curves represent the average results from four to nine separate experiments.

cells of newborn and 1-week-old animals at high agonist concentrations and that this is true in either type of incubation solution (Fig. 5). By contrast the plateau values of the Ca^{2+} response were generally not significantly different in immature and mature cells, either in the presence or absence of external (medium) Ca^{2+} (Fig. 6), or when Ca^{2+} was added back at the end of experiments performed in Ca^{2+}-free solutions.

$^{45}Ca^{2+}$ Uptake and Release from Intracellular Stores. Although there was considerable day-to-day variability in the amount of ^{45}Ca taken up by different permeabilized cell preparations, it was clear that adult cells took up substantially more Ca^{2+} into ATP-dependent stores than cells from either of the two younger groups (steady state levels of ATP-dependent uptake were 5.80 ± 0.70, 6.25 ± 0.55, and 14.25 ± 0.75 nmol/mg protein for cells of 1-day-old, 1-week-old, and adult animals, respectively). There was also a small, but significant, amount of Ca^{2+} taken into non-ATP-dependent stores, and this component was greatest in the cells of the 1-week-old animals (0.987 ± 0.044, 1.58 ± 0.19, 0.880 ± 0.042 nmol/mg protein for cells of 1-day-old, 1-week-old, and adult animals, respectively). The biologic relevance of this non-ATP-dependent uptake is not known, but since the IP_3-sensitive stores are believed to accumulate Ca^{2+} in an ATP-dependent manner, the ATP-independent component of the uptake was subtracted from the steady state values

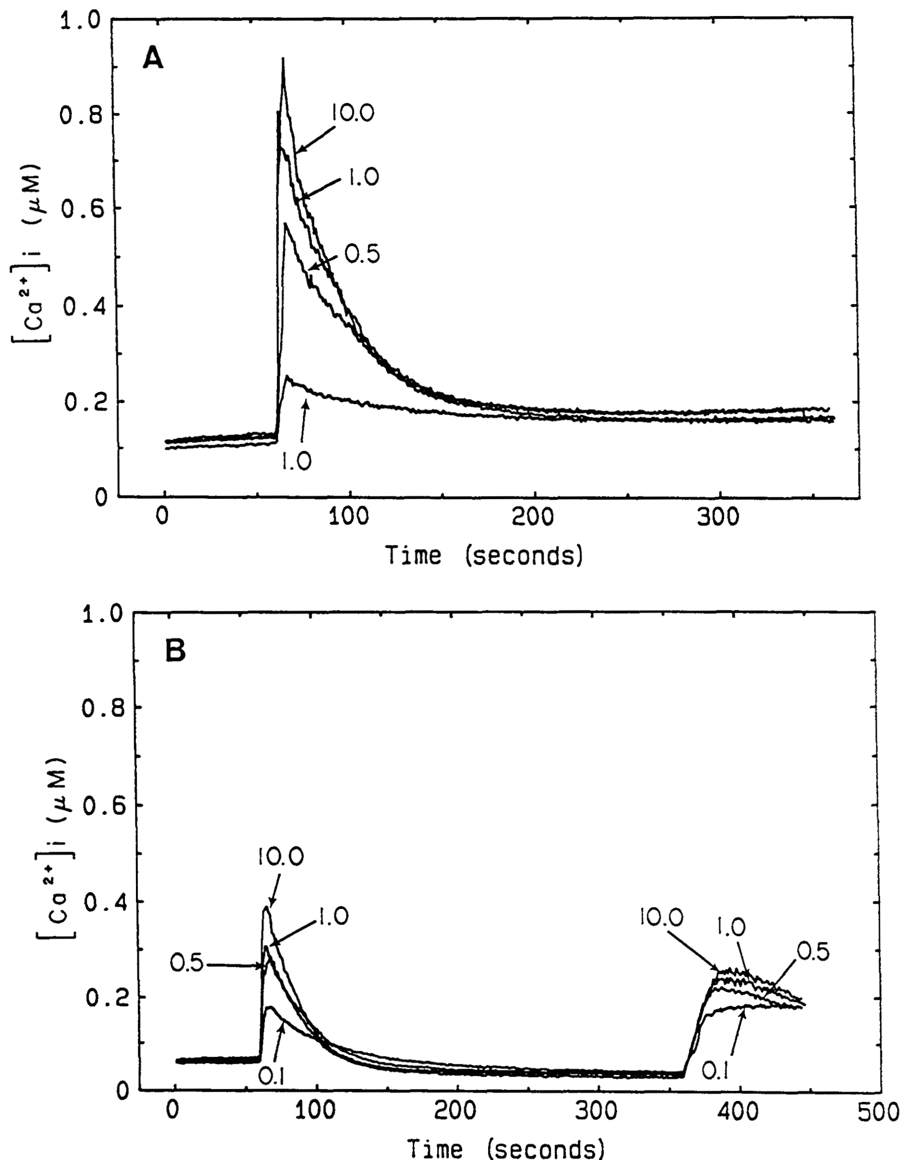

Figure 4. Intracellular Ca^{2+} was measured in cells of 1-week-old animals with fura-2 as described in Materials and Methods, in buffers containing 1 m*M* $CaCl_2$ (A) or 0.1 m*M* EGTA and no added $CaCl_2$ (B). Cells were exposed to the following concentrations of acetylcholine: 0.1 μ*M* (curves marked 0.1), 0.5 μ*M* (curves marked 0.5), 1.0 μ*M* (curves marked 1), and 10 μ*M* (curves marked 10). $CaCl_2$ (1 m*M*) was added to the curves in 4B at 360 sec. Curves represent the average results from four to five separate experiments.

achieved 10 min after ATP addition. To compensate for the day-to-day variability, the data for IP_3-induced efflux of Ca^{2+} are presented as percentage of ATP-dependent steady state values, and these data show important differences among cells of the 1-day-old, 1-week-old, and adult animals (Fig. 7). In mature cells, a much steeper dose-response relationship was seen, with a clear maximal response at the three highest doses, so that less than 20% tracer was released by 0.1 μ*M* IP_3, but between 50% and 60% at the earliest time point (i.e., 30 sec) by 0.5–10 μ*M* IP_3 (Fig. 7A). In contrast, cells of 1-day-old and 1-week-old animals had a more graded response to the various agonist concentrations (Fig. 7, B and C). These results are also plotted as a function of IP_3 dose in Figure 8. There is a clear shift to the right in the dose-response relationship of cells from the early postnatal animals, and the differences from the responses of cells of adult animals are highly significant: $P < 0.001$ for 1-week-old rats at 0.5 μ*M* and 1 μ*M* IP_3 and $P < 0.02$ for 1-day-old rats at 0.5 μ*M* IP_3.

Discussion

The experiments described above were designed as a comparative study of some aspects of the muscarinic signal transduction pathway in immature and mature salivary cells. Previous evidence had indicated that the immature cells of early postnatal animals had comparable, or even somewhat increased, Ca^{2+} responses (measured with fura-2) to those of mature cells when exposed to an optimal concentration of acetylcholine

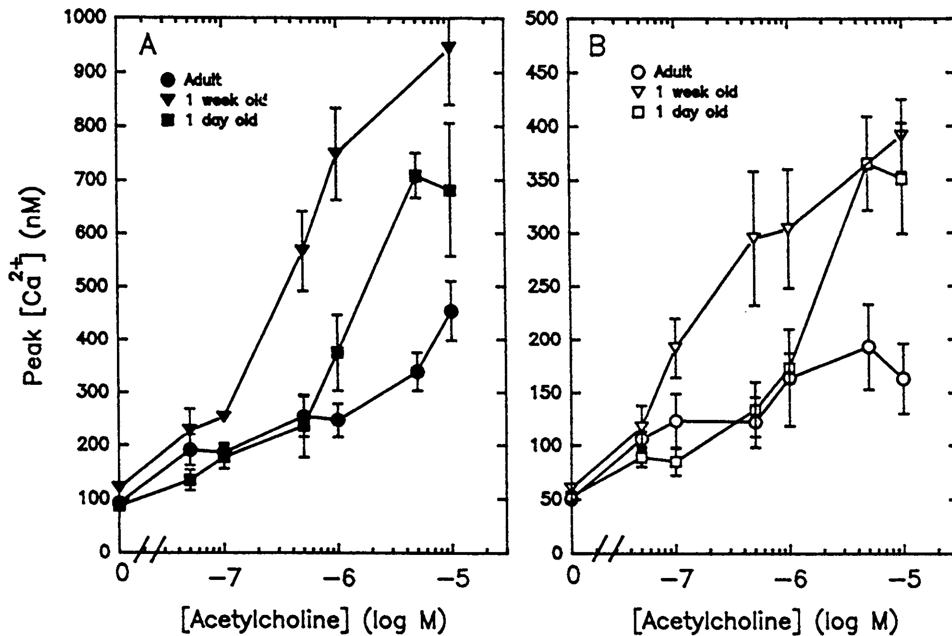

Figure 5. The initial peak Ca^{2+} responses of submandibular cells from 1-day-old, 1-week-old, and adult animals in Ca^{2+}-containing (A, solid symbols) and Ca^{2+}-free (B, open symbols) buffers are shown as a function of acetylcholine concentration. Circles: adults; triangles: 1-week-old; and squares: 1-day-old. Data shown are the mean and SE of data shown in Figures 2–4.

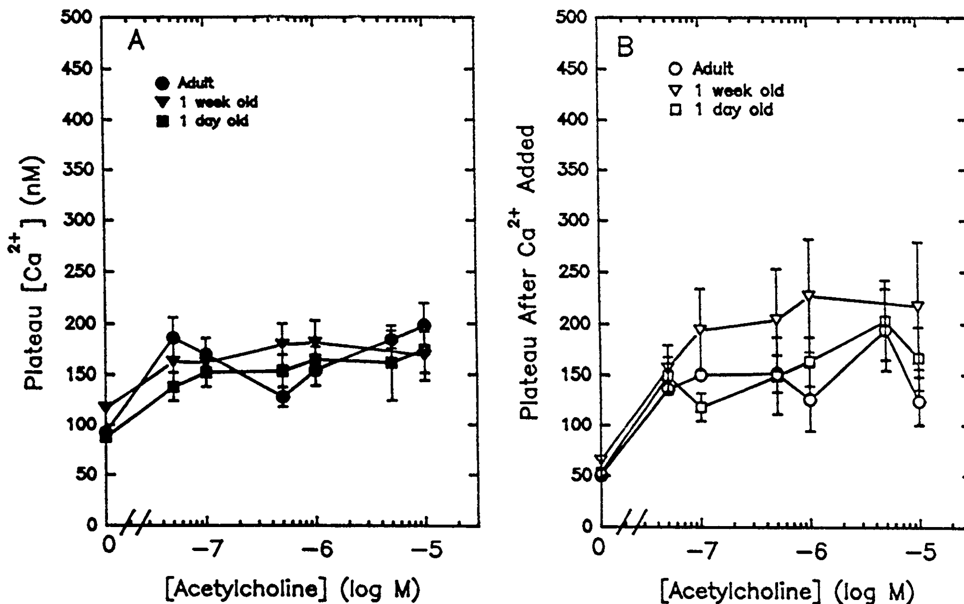

Figure 6. The plateau phase (240–270 sec) $[Ca^{2+}]_i$ responses of submandibular cells from the three age groups incubated in Ca^{2+}-containing buffer (A) are shown as a function of acetylcholine concentration. (B) The open symbols show the plateau levels attained when 1 m*M* $CaCl_2$ was added to the cell suspensions after exposure to agonist in Ca^{2+}-free buffers for cells of the different age groups. Data shown are the mean and SE of data from the experiments shown in Figures 2–4. Circles: adults; triangles: 1-week-old; and squares: 1-day old.

(12). The results reported here expand on this observation and demonstrate dose- and age-related differences in the formation of IP_3 and in the Ca^{2+} responses of cells derived from early postnatal animals.

Formation of inositol trisphosphate, an important element of the muscarinic signal transduction pathway, was comparable in cells of the three age groups studied after exposure to acetylcholine concentrations smaller than 1 μM. At higher agonist concentrations, however, the cells of 1-day-old and 1-week-old animals generated significantly more IP_3 than those of adult animals. The reasons for this enhanced response are not clear, but could be related to differences in receptors, in membrane phospholipid turnover, or in the coupling between the two in the early stages of postnatal development. These possibilities require further investigation,

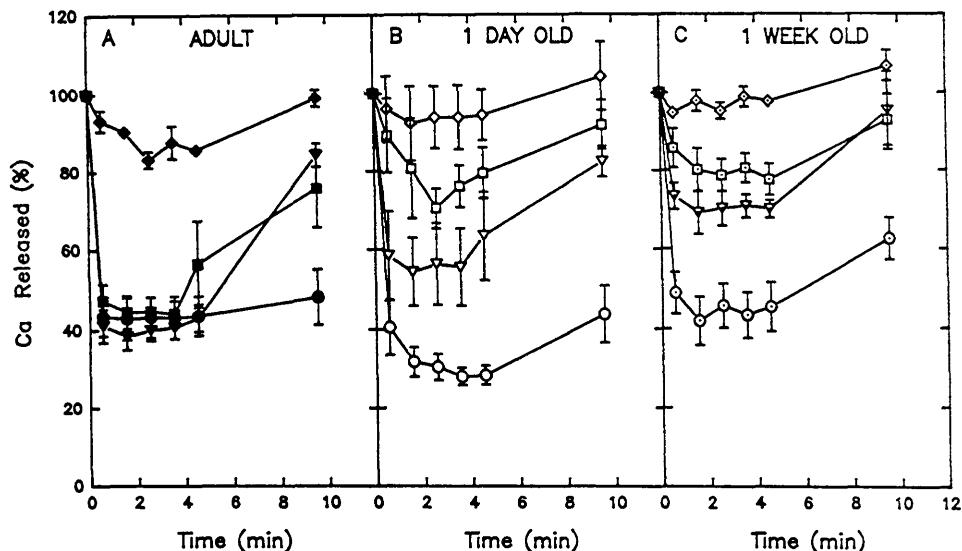

Figure 7. IP_3-induced efflux of $^{45}Ca^{2+}$ was determined in permeabilized cells loaded with the tracer in the presence of mitochondrial inhibitors as described in Materials and Methods. The following concentrations of IP_3 were used: diamonds, 0.1 μM; squares, 0.5 μM; triangles, 1 μM; and circles, 10 μM. (A) Cells from adult animals; (B) cells from 1-day-old animals; and (C) cells from 1-week-old animals. Data represent the mean and SE of three to five experiments.

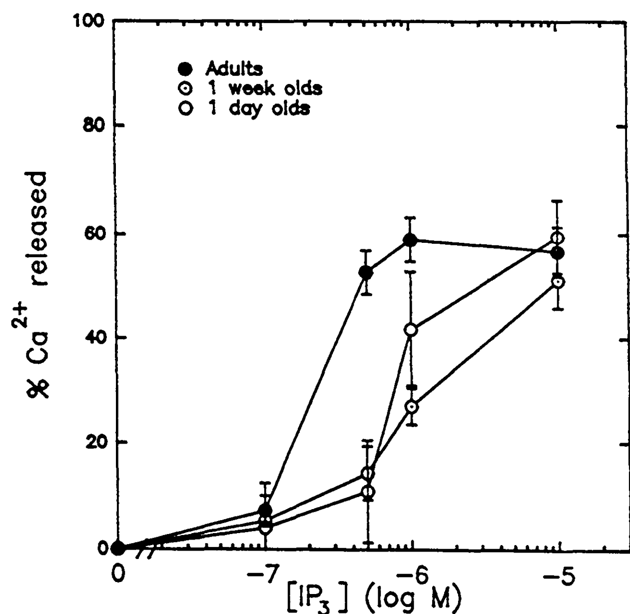

Figure 8. The mean and SE of the initial (30 sec) extent of IP_3-induced ^{45}Ca release from cells of the various age groups are replotted from Figure 7 as a function of the concentration of IP_3. Solid circles, adults; open circles, 1-day-old; and dotted circles, 1-week-old.

but our results clearly demonstrate a significant shift in the dose-response relationship for IP_3 generation in the immature salivary cells of early postnatal animals.

Inositol trisphosphate acts as an intracellular signal to induce the release of Ca^{2+} stored inside the cell (13, 14). Since the immature salivary cells of young rats generated more IP_3 than mature cells on exposure to the higher concentrations of acetylcholine, one would expect a larger Ca^{2+} response in these cells at these levels of stimulation. Our measurements of intracellular Ca^{2+} with fura-2 in Ca^{2+} confirmed this expectation. The initial peaks in the Ca^{2+} response observed in the absence of external Ca^{2+} represent release of stored Ca^{2+} and these peaks were significantly higher in the cells of 1-day-old and 1-week-old animals exposed to the higher agonist concentrations. This finding suggests that the immature cells possess adequate numbers of IP_3 receptors and an IP_3-sensitive store capable of releasing a substantial amount of Ca^{2+} upon stimulation. Further analysis of the data, however, revealed a poor correlation between IP_3 production and Ca^{2+} release from internal stores in the cells of early postnatal animals. For example, exposure to 1 μM acetylcholine resulted in comparable IP_3 production in cells of 1-week-old and adult animals, but the peak Ca^{2+} response in Ca^{2+}-free buffers was significantly higher in the former cells. This analysis suggests differences in either the sensitivity of the internal Ca^{2+} stores to IP_3 or in the stores themselves of mature and immature salivary cells. We examined the first possibility and found differences in the release of Ca^{2+} from nonmitochondrial pools in permeabilized cells of the three age groups exposed to various doses of IP_3. However, the differences were in the opposite direction from those that would be required to account for the increased intracellular Ca^{2+} response in neonatal cells observed in the fura-2 experiments. Cells from adult animals took up more Ca^{2+} into intracellular stores, and the dose-response relationship to IP_3 was shifted to the right in the immature cells (Fig. 8), suggesting that the sensitivity of Ca^{2+} stores to IP_3 actually increases with age. Two

other possible explanations may account for the greater peak Ca^{2+} response (fura-2 studies) observed in the younger cells in Ca^{2+}-free buffers, at agonist concentrations producing similar amounts of IP_3. First, the IP_3 measurements represent content, normalized to cellular protein, not concentration, and it may be that in cells from the younger animals, the IP_3 is actually concentrated in a smaller volume, resulting in a greater concentration. Second, it should be noted that the $^{45}Ca^{2+}$-release assays in permeabilized cells were performed under conditions in which the cytoplasmic face of the Ca^{2+} stores is exposed to solutions containing a constant concentration of Ca^{2+} buffered with EGTA, and thus differs from the physiologic condition in which the localized concentration of Ca^{2+} may change severalfold during the release of Ca^{2+} from its stores. The interaction of IP_3 with its receptors and the effects of Ca^{2+} on IP_3 binding and Ca^{2+} channel activation show complex regulation in other cell systems (20–25). These aspects of signaling in the salivary acinar cells will require further investigation.

Increased initial peaks in the Ca^{2+} responses were also observed in immature salivary cells incubated in Ca^{2+}-containing solutions and the differences in the initial peaks seen in Ca^{2+}-containing and Ca^{2+}-free incubation solutions were greater in immature cells, suggesting a greater initial influx of external Ca^{2+} in these cells. However, the plateau values of the fura-2 responses in the three cell populations were comparable at all agonist concentrations. These plateau values in Ca^{2+}-containing solutions are higher than the resting Ca^{2+} levels, and are thought to result from the sustained entry of Ca^{2+} in the continued presence of agonist. Also, the levels of Ca^{2+} attained when Ca^{2+} was added back to cell preparations exposed to acetylcholine in Ca^{2+}-free solutions were similar in the three age groups. These two types of observations do not support the view of an enhanced Ca^{2+} entry in the immature cells, and the reason for the differences in the initial peaks observed in the presence and absence of Ca^{2+} could represent differences in the release of Ca^{2+} from storage sites that are rapidly depleted in the absence of extracellular Ca^{2+}.

The increased generation of IP_3 and the enhanced Ca^{2+} signal at higher levels of stimulation, plus the suggestion of a different sensitivity of the internal Ca^{2+} stores to IP_3, suggest important functional differences in the muscarinic signaling pathway of developing salivary cells of early postnatal rats. Together with previous evidence of deficient Ca^{2+}-regulated efflux of K^+ and Cl^- in the immature cells (6, 9, 11, 12), the results of the present study indicate that elements of the muscarinic signaling pathway vary as the cells mature during postnatal development. The IP_3/Ca^{2+} signaling pathway is known to be involved in the responses of many cells to various growth factors (reviewed in Refs. 26 and 27). This pathway may thus be initially associated with the demands of morphologic and functional development in salivary cells, and later change to a role in secretion. This is consistent with the functional dissociation between an increased Ca^{2+} signal and the reduced activation of K^+ and Cl^- channels in the immature salivary cells (9).

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