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Receptor Control of Calcium Influx in Parotid Acinar Cells

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SUMMARY

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The effects of various agonists and antagonists on Ca^{++} influx in dispersed rat parotid acinar cells were investigated. The cell preparation was first characterized. The cells maintained normal ultrastructure and physiological content of Na^+ and K^+, and secreted amylase or K in response to appropriate secretagogues. Uptake of ^{45}Ca was stimulated by carbachol, epinephrine, substance P and isoproterenol. Isoproterenol, but not carbachol, stimulated uptake of [^{14}C]sucrose and caused a decrease in protein content per aliquot of cells. These latter observations suggest that the increased Ca^{++} uptake due to *beta* adrenergic stimulation may, at least in part, result from pinocytosis. The stimulated ^{45}Ca uptake due to either carbachol or substance P was blocked by 3.0 mM $CoCl_2$. Procaine (1.0 mM), however, blocked the response to carbachol but not substance P. These results support an hypothesis suggesting that Co^{++} blocks inward transport of Ca^{++} while procaine interferes with the transduction mechanism linking the muscarinic receptor to the Ca^{++} channel.

INTRODUCTION

The rat parotid gland has proved an extremely useful tool for studies of Ca^{++} and cell function because of the diverse receptor mechanisms and Ca^{++}-mediated phenomena that occur (1). Stimulation of muscarinic, *alpha* adrenergic or peptide receptors leads to an increase in membrane permeability to K^+ and a limited discharge of amylase *via* exocytosis (2-5). For these receptors, both responses (release of K^+ and discharge of amylase) are dependent, at least in part, on the presence of extracellular Ca^{++} (1). Stimulation of *beta* adrenergic receptors, or application of derivatives of cyclic adenosine-3',5'-monophosphate (cyclic AMP) produces a substantial release of amylase with little or no effect on membrane permeability to K^+ (2). This response does not require extracellular Ca^{++}, but the dependence on cellular Ca^{++} stores noted suggests that Ca^{++} released internally might mediate the effects (6).

Recently, a number of investigators have reported the preparation of viable, functional dispersed acinar cells from the rat parotid gland (7-11). Some investigators have observed that certain secretagogues will stimulate influx of ^{45}Ca into the acinar cells. However, results obtained by different investigators have differed. Miller and Nelson (12) found that *beta* agonists and derivatives of cyclic AMP caused sizeable increases in ^{45}Ca influx whereas muscarinic or *apha* adrenergic agonists caused less (though significant) increases. Kanagasuntheram and Randle (10) and Koelz *et al.* (11) reported quantitatively similar effects of muscarinic or *alpha* adrenergic stimulation, but found no effect of *beta* adrenergic stimulation on the influx of ^{45}Ca.

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The purpose of this investigation was two-fold: First, we have attempted to shed some light on the controversy regarding *beta* adrenergic stimulation of Ca^{++} influx by measuring ^{45}Ca influx, electrolyte levels (as an index of viability), and secretory responses simultaneously in a well-characterized, dispersed acinar cell preparation. Second, we have also used this preparation to test some recent predictions on the pharmacology of Ca^{++} influx in the parotid gland (13).

MATERIALS AND METHODS

Preparation of parotid cells. Male Wistar rats (wt. 150–250 g) were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and parotid glands removed into a small volume of a physiological Ringer solution of the following composition (millimolar concentrations): NaCl, 120; KCl, 5.0; $MgCl_2$, 1.2; CaCl, 1.0; β-hydroxybutyrate sodium, 5.0; tris(hydroxymethyl)aminomethane, 20.0 and buffered with HCl to pH 7.4 at 37°C. The tissue was dispersed by the method of Kanagasuntheram and Randle (10) except that only 2 rats were used and the quantity of enzymes reduced by half.

The cell pellet obtained in the isolation procedure was usually resuspended in 4 ml Ringer's medium containing 2% bovine serum albumin (BSA) to give a cell concentration of approximately 10^7 cells/ml. Cells were observed with both light and phase-contrast microscopy; the preparation was usually between 60–80% single cells (predominately acinar) with the remainder in clumps of 3 to 10 cells. Greater than 95% viability was observed as determined by trypan blue exclusion.

Measurement of ionic composition, amylase content, and ^{45}Ca uptake of cells. Aliquots of cell suspension were transferred to 5 ml plastic vials, incubated at 37° and continually gassed with 100% O_2. The incubation medium had the composition given above with 0.5% BSA added. Cells were usually allowed to pre-equilibrate for at least 30 min before starting experiments. At various times, a 0.1 ml aliquot of cells was removed into 1 ml of cold 320 mM sucrose and centrifuged for 1 min in a Eppendorff microcentrifuge at 11,000 rpm. The supernatant was discarded and the pellet rinsed once with cold sucrose (i.e., the tubes were filled with cold sucrose and quickly emptied without resuspending the pellet). In ^{45}Ca uptake experiments, 1 mM $LaCl_3$ was added to the cold sucrose to reduce the amount of surface bound ^{45}Ca on the cells. Time required for the $LaCl_3$ to displace surface calcium was tested by placing aliquots of cells into centrifuge tubes containing cold sucrose + $LaCl_3$ and delaying 2, 5 and 10 min before centrifuging. Binding of ^{45}Ca (% of control, or 0 time sample) was found to be 84, 88, and 76%, respectively. In subsequent experiments, cells were allowed to stand in sucrose-$LaCl_3$ for 2 min before centrifuging. Content of ^{45}Ca measured with sucrose-$LaCl_3$ averaged 72% of the content measured with sucrose alone for separation.

Following centrifugation, the pellet was resuspended in distilled, deionized water and lysed by rapid freezing and thawing. Aliquots were taken for determination of protein, electrolytes and amylase, and radioisotope uptake where applicable. Cell protein content was determined by the method of Lowry *et al.* (14). Electrolyte content was determined with a Perkin-Elmer Atomic Absorption Spectrophotometer. Electrolytes were expressed as nmoles/μl cell H_2O where cell H_2O was calculated as 4.3 μl/mg protein.[2] Cell amylase content was determined by the method of Bernfeld (15) and expressed as the percentage of amylase content in the first sample taken. Amylase content of freshly prepared cells was 125 ± 3 units/mg cell protein in 18 determinations. Uptake of radioisotope was determined by drying an aliquot of the lysed cell preparation on a planchet and counting in a Searle planchet

[2] The factor was calculated as follows. Slices of rat parotid gland were found to contain 0.10 ± 0.01 mg protein/mg wet weight (unpublished observation). The slices also contain 0.43 μl cell H_2O/mg wet weight (3). Thus, 0.43 μl H_2O/mg + 0.1 mg protein/mg = 4.3 μl H_2O/mg protein. Similarly, since the extracellular space of the slices is 0.3 ml/g (3), then 0.7 g cells/g + 0.1 g protein/g = 7 g cells/g protein. These are approximations of course but facilitate interpretation of the data from a physiological standpoint without affecting the statistical assessment of the results.

counter. Uptake was expressed as ml/g of cells where cell weight was calculated as 7 times the cell protein content.[2] An aliquot of cell-free supernatant was taken for determination of media radioactivity.

Media volume trapped by the cell pellet was determined by adding an aliquot of cells to cold sucrose containing 0.1 μCi/ml [^{3}H]inulin, centrifuging and rinsing with cold sucrose as described above. Thirty-two determinations yielded an average trapped volume of 0.102 ± 0.006 ml/g, calculated as for ^{45}Ca. In some preliminary experiments, cells were also separated from media with Millipore filters (pore size, 1.2 μ) and by centrifuging through Dow-Corning 510 and 550 fluid, a water-impermeable barrier of silicon oil. No differences in results were noted, but reproducibility was superior with the cold sucrose centrifugation in our laboratory. Release of ^{86}Rb from dispersed cells was determined by placing a small aliquot of cells in a basket constructed of 10 μm nylon mesh and monitoring efflux as previously described for parotid gland slices (5).

For electron microscopical examination, a 10 μl sample of the dispersed cell preparation (incubated for 1 hr) was added to 1 ml of cold 1.5% glutaraldehyde in 0.1 M Na cacodylate (pH 7.3). The suspension was centrifuged briefly in a microcentrifuge so that the cells were sticking to the sides of the microcentrifuge tube. Fixation was continued overnight in the cold (16–17 hr), according to the method of Cope and Williams (16). The next morning the sample was washed 8 times with cold 0.15 M Na cacodylate buffer (80 mM sucrose, pH 7.3) over a two hour period. Postfixation was carried out in cold 1% osmium tetroxide in 0.15 M Na cacodylate buffer (plus 30 mM sucrose, pH 7.3) for 1.5 hours, after which the cells were washed twice in the cacodylate buffer at room temperature (15 min each wash) and twice in 20% ethanol (15 min each). (The cells were pelleted in a microcentrifuge for 1 min in the buffer and resuspended and pelleted for every step up to infiltration.) The cells were stained *en bloc* in 2% aqueous uranyl acetate for 15 min at room temperature and then dehydrated in a graded series of ethanol solutions starting with 50% and embedded in Spurr's low viscosity resin (17). Thin sections were cut on a Sorval Porter-Blum MT-1 Ultramicrotome with glass knives, stained for 1 hr in saturated uranyl acetate at 60° and for 1 min in 0.2% lead citrate (18), and examined in a Philips EM 300 electron microscope.

Statistical analyses were performed with one- or two-way analysis of variance. Maximum acceptable significance level for p was 0.05. Dispersions about the mean in graphic summaries represent one standard error of the mean.

Materials. Trypsin (type III), trypsin inhibitor (type II-S), collagenase (type II) and bovine albumin (fraction V) were obtained from Sigma Chemical Co., St. Louis, Mo., as were all agonists and antagonists with the exception of substance P, which was purchased from Bachem Chemicals, Marina del Rey, Ca. The ^{45}Ca, [^{14}C]sucrose and [^{14}C]inulin were obtained from New England Nuclear, Boston, Mass. Glutaraldehyde (8%, EM grade) osmium tetroxide crystals and sodium cacodylate were purchased from Electron Microscopy Sciences, Fort Washington, Pa.

RESULTS

*The cell preparation.*The morphology of cells, isolated in our laboratory with the procedure of Kanagasuntheram and Randle (10), was well preserved. Occasional vacuolation was noted but this was not detected by phase contrast microscopy and may result from fixation. Fig. 1 shows a typical isolated acinar cell used in these experiments.

The levels of Na^+ and K^+ in the incubated cells are shown in Fig. 2. The concentration of K^+ was 2–3 times the concentration of $Na.^+$ The values for both ions were slightly lower than those calculated for intracellular water previously (3); however, the ratio of K^+ to Na^+ is somewhat higher. By comparison to other cell preparations, the K^+ concentration is lower than that reported by Kanagasuntheram and Randle (10) and similar to that reported by Mangos *et al.* (7). Of preceding reports, only that of Mangos *et al.* (7) report Na^+ levels, and our

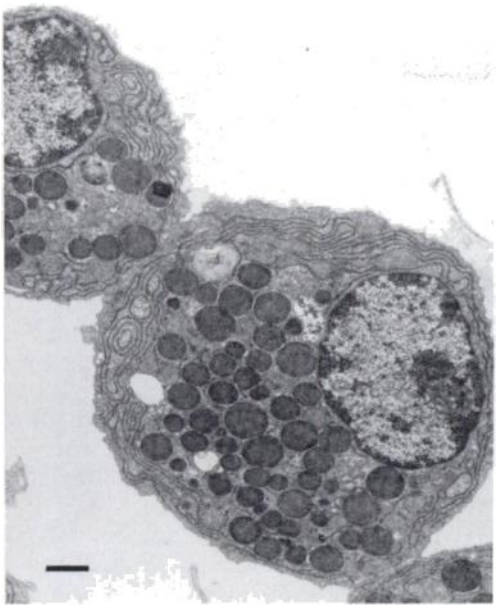

FIG. 1. *A typical acinar cell from the dispersed cell preparation*

Most of the cells in the preparation are single, some are attached in clumps of three or more (of which this is an example). The point of attachment is at the junctional complex between the acinar cells, leaving the rest of the limiting cell membrane exposed. Cellular fine structure has been preserved as in whole tissue; several small vacuoles may be present. Bar represents 1 μm.

values appear to be somewhat lower (by about one half).

The secretory response of the cells was maintained following isolation (Figs. 3 and 4). No significant loss in cell amylase occurred in the absence of stimulation. Isoproterenol (10 μM) caused a secretion of amylase at a rate of about 1%/min (Fig. 3), a rate that is comparable to that obtained with slices. Mangos *et al.* (8), Kanagasuntheram and Randle (10), and Miller and Nelson (12) report comparable rates. The preparation utilized by Koelz *et al.* (11) demonstrated a 2-fold increase in secretion rate due to 10 μM epinephrine.

The K^+ release response with isolated cells has been previously tested only by Mangos *et al.* (8, 9). They found a transient net loss of K^+ from the cells following muscarinic or *alpha* adrenergic stimulation. We have followed unidirectional efflux of ^{86}Rb from cells with a procedure similar to that employed with slices (5, 13, 19). As shown in Fig. 4, K^+ release dependent on, and independent of the external Ca^{++} could be demonstrated with carbachol (10 μM) and with substance P (0.1 μM). These patterns of efflux are similar to those reported previously for slices (5, 13, 19).

Influx of ^{45}Ca. Figure 5 depicts the time course of uptake of ^{45}Ca by the cell preparation. The dashed line represents that volume of incubation medium estimated to be trapped when the cells are centrifuged. Analysis of variance (two-way) revealed that both isoproterenol and carbachol significantly elevated ^{45}Ca uptake. After correcting for trapped volume, the relative stimulation would appear to be maximal at about 10 min. Note that these data are expressed as apparent volumes of distribution (ml/g, see MATERIALS AND METHODS), but may be converted to μmol/g by multiplying by the bath Ca^{++} concentration, 1.0 μmol/ml. The increase due to carbachol and isoproterenol at 10 min represents about 1.0 nmol/mg protein. Though the methods differ somewhat, this is within the range of values reported by Kanagusuntheram and Randle (10) for carbachol and by

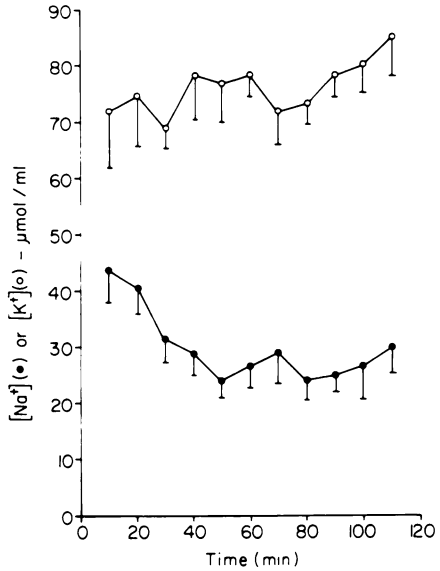

FIG. 2. *Electrolyte levels in dispersed rat parotid acinar cells*

Cells were prepared and Na^+ and K^+ content measured as described in MATERIALS AND METHODS. Each value is the mean of three determinations ± 1 S.E.M.

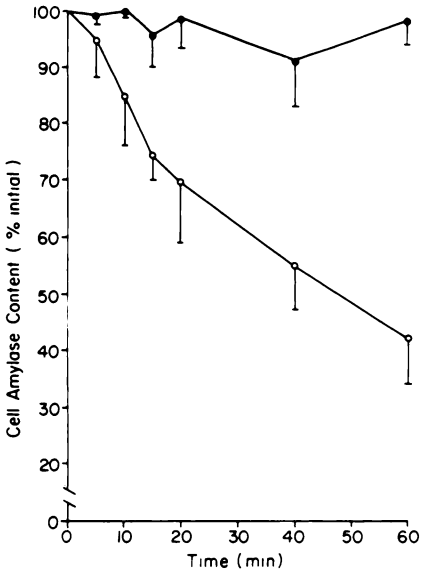

FIG. 3. *Amylase content in dispersed parotid acinar cells in the absence* (●) *and in the presence* (○) *of 10 μM isoproterenol*

Each value is the mean of three determinations ± 1 S.E.M.

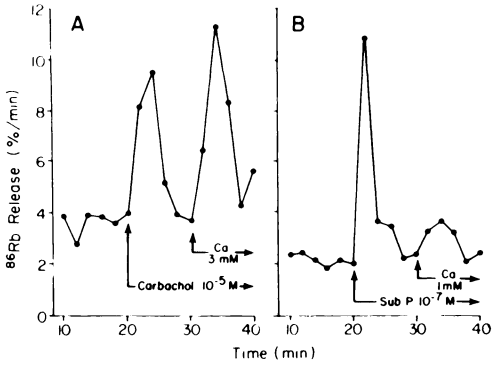

FIG. 4. *Release of K^+ (^{86}Rb) from dispersed parotid cells*

Cells were incubated for 30 min in Ringer medium containing 2-10 μCi/ml ^{86}Rb. The cells were placed in a small basket constructed of nylon mesh (10 μm pore size) and serially transferred through 20 two-minute incubations. Released ^{86}Rb was determined as well as that remaining in the cells, so that first order rate coefficients could be calculated (5). A and B, no added Ca + 0.1 mM EGTA, 0-30 min. A: 10 μM carbachol, 20-40 min; 3.0 mM $CaCl_2$, 30-40 min. B: 0.1 μM substance P, 20-40 min; 1.0 mM $CaCl_2$, 30-40 min.

Miller and Nelson (12) for isoproterenol and carbachol.

Uptake of [^{14}C]sucrose was also measured to determine whether pinocytosis might account for some of the observed uptake. (Note that these data represent the quantity of sucrose taken up from the incubation medium, after various times of incubation, while trapped volume was determined by adding labeled material to the cold sucrose.) With or without the addition of drugs, uptake of [^{14}C]sucrose proceeds at a slow but significant rate (Fig. 6), in agreement with earlier observations with slices (3). Whether this uptake represents a small but finite membrane permeability to sucrose or pinocytotic uptake cannot be determined from these experiments. Whatever the mechanism, carbachol did not significantly affect uptake of [^{14}C]sucrose, while isoproterenol caused a significant elevation (Fig. 6).

The protein content per aliquot tended to fall slightly throughout these experiments. Figure 7 shows data pooled from the experiments in Figs. 5 and 6. After one hour, the protein per aliquot was 92-93% of its original (2 min) value in control experi-

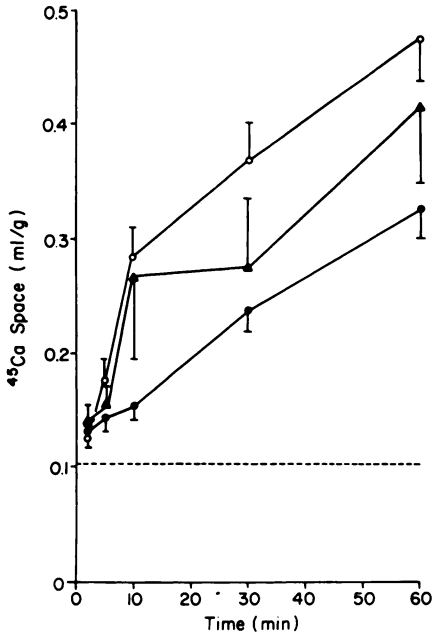

FIG. 5. *Uptake of ^{45}Ca by dispersed parotid cells in the absence of drugs* (●, *n* = 8) *and in the presence of 10 μM isoproternol* (○, *n* = 5) *or 0.1 mM carbachol* (▲, *n* = 3)

The dashed line indicates the estimated trapped volume of Ringer (see MATERIALS AND METHODS). Each value is the mean ± 1 S.E.M.

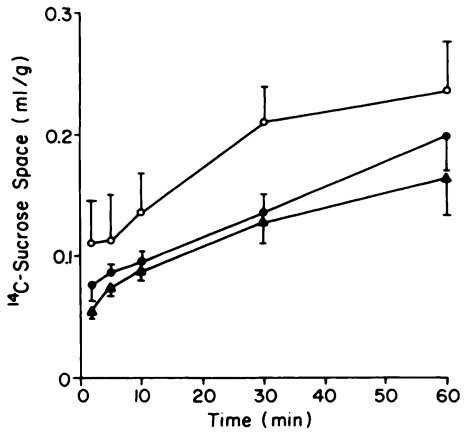

FIG. 6. *Uptake of [^{14}C]sucrose by dispersed parotid cells in the absence of drugs* (●, *n* = 6), *and in the presence of 10 μM isoproterenol,* (○, *n* = 3) *or 10 μM carbachol* (▲, *n* = 3)

Each value is the mean ± 1 S.E.M.

ments and with carbachol addition. With the cells treated with isoproterenol, the protein content in one hour fell to less than 80% of the initial (2 min value). This is presumably due to the secretion of amylase resulting in a decrease in protein content per cell. In support of this, Na^+ and K^+ contents, expressed per mg of protein, were significantly increased after 30 or 60 min incubation in the presence of isoproterenol (data not shown).

Pharmacology of ^{45}Ca influx. Fig. 8 summarizes experiments in which ^{45}Ca uptake (10 min) due to each of several agonists was determined. As before, carbachol (0.1 mM and 10 μM) and isoproterenol (10 μM) significantly elevated ^{45}Ca uptake. Substance P (0.1 μM) and epinephrine (0.1 mM) also significantly elevated ^{45}Ca uptake. The effect of epinephrine was significantly greater than that of isoproterenol, in agreement with previous observations by Miller and Nelson (12).

In an earlier report (Marier *et al.*, 1978) we noted that Co antagonized Ca-dependent responses to carbachol and substance P, while procaine affected only the muscarinic response. Thus, the effects of procaine (1.0 mM) and $CoCl_2$ (3.0 mM) on uptake of ^{45}Ca due to carbachol or substance P were determined. The results of these experiments are shown in Fig. 9. In the presence of 1.0 mM procaine, carbachol failed to stimulate ^{45}Ca uptake while the effect of substance P was unaltered. With 3.0 mM $CoCl_2$, however, neither agonist significantly increased ^{45}Ca uptake.

DISCUSSION

Our data on this particular cell preparation confirm those of the originators (10) in demonstrating that after isolation, the cells maintain reasonable morphological integrity, high cellular K^+ content, and responsiveness to *beta* adrenergic stimulation. We have extended the characterization to show that cellular Na^+ content is low and that typical K^+ release responses can be obtained. Thus the preparation seems to have retained the relevant functional characteristics of non-enzymatically-treated tissue. One other preparation of dispersed parotid acinar cells, described by Mangos *et al.* (7–9), has been similarly characterized and shown to maintain these same functions.

With this preparation, we observed a significant stimulation of ^{45}Ca uptake due to isoproterenol and carbachol. The results with carbachol confirm those of Kanagasuntheram and Randle (10), Miller and Nelson (12) and Koelz *et al.* (11). They also confirm the earlier observations with slices (6, 20). Stimulation of ^{45}Ca uptake by iso-

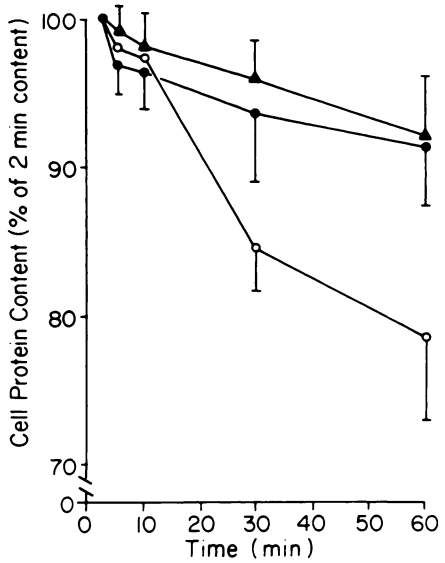

FIG. 7. *Protein content per aliquot of cells, from the experiments in Figs. 5 and 6*

●, control, *n* = 14; ○, isoproterenol, *n* = 8; ▲, carbachol, *n* = 3. Each value is the mean ± 1 S.E.M.

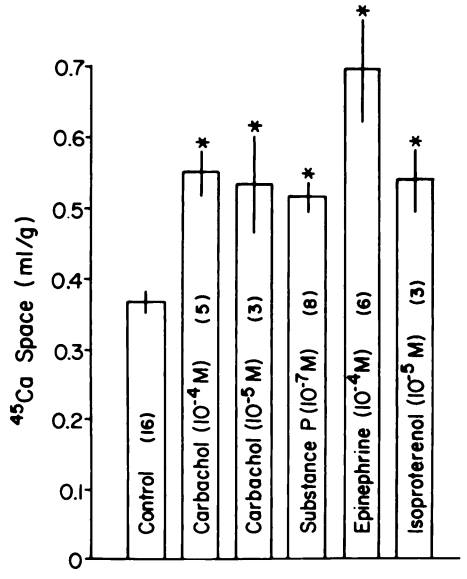

FIG. 8. *Uptake of ^{45}Ca in response to various agonists*

Uptake time was 10 min. Each value is the mean ± 1 S.E.M. The numbers in parentheses indicate the number of measurements. Values significantly different from control are indicated by (*).

proterenol was not observed in our earlier experiments with slices (20), nor was it detected by the originators of this cell preparation, Kanagasuntheram and Randle (10). With a preparation less extensively characterized, Koelz *et al.* (11) failed to detect effects of isoproterenol on ^{45}Ca uptake by parotid cells. Amylase secretion also responded only weakly to isoproterenol in their preparation. Miller and Nelson (12), using cells prepared according to Mangos *et al.* (7), obtained marked stimulation of ^{45}Ca uptake with isoproterenol.

The reasons for these discrepancies are not clear. Of the three reports in which well characterized cells were used, two reported stimulation due to *beta* adrenergic agonist (12, this report) and one reported no effect (10). It is also disturbing that two of the conflicting reports utilized the same method of cell preparation (this report, 10).

The effects of isoproterenol on [^{14}C]sucrose uptake and on cell protein add some confusion to the interpretation of the ^{45}Ca uptake data. If the protein content per cell decreases with time, then the influx per cell at later times (30 and 60 min) is slightly overestimated in Fig. 5. Such a correction would not appear to be significant at 10 min, however, where the greatest relative effect of isoproterenol was observed.

The increased [^{14}C]sucrose uptake due to isoproterenol at 10 min suggests that extracellular medium may be taken up due to pinocytosis that presumably occurs with exocytosis. If ^{45}Ca is taken up in the same proportion to sucrose as occurs in the medium, then the amount suggested by Fig. 6 is insufficient to account for the ^{45}Ca taken up in Fig. 5. Accordingly, some stimulation of Ca^{++} influx would occur. If, however, membrane recaptured after exocytosis could bind Ca^{++}, then all of the extra ^{45}Ca uptake due to isoproterenol could occur by this route. Regrettably, an experimental tactic to distinguish these possibilities is not presently available. The earlier negative data in slices (6) might support the pinocytotic mechanism since, in slice preparations, substances added to the bathing medium do not gain access to the luminal membrane (21).

Such considerations do not complicate interpretation of the effects of carbachol. As stated above, several investigators agree on the cholinergic stimulation of ^{45}Ca uptake. The data in Figs. 6 and 7 suggest that

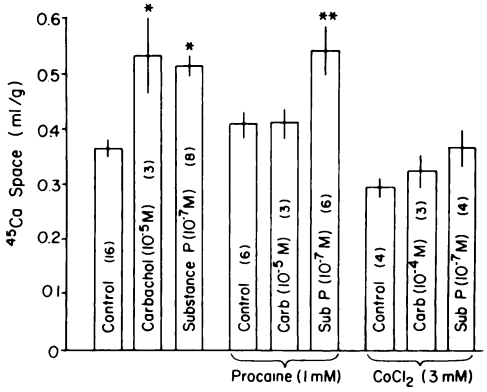

FIG. 9. *Uptake of ^{45}Ca in response to carbachol or substance P as affected by procaine (1 mM) or $CoCl_2$ (3 mM)*

The antagonists were present during the uptake period (10 min) and during the 30 min pre-equilibration period. Each value is the mean ± 1 S.E.M. (*) indicates values significantly greater than control; (**) indicates values significantly greater than control in the presence of 1 mM procaine.

changes in protein content or stimulation of pinocytosis do not contribute to these measurements. Further, a stimulation of Ca^{++} influx by carbachol is a mechanism consistent with other physiological aspects of the carbachol response. This is not so clearly the case for isoproterenol.

This is the first demonstration that Ca^{++} influx is stimulated by substance P (Figs. 8 and 9). Previously it was suggested that substance P and carbachol, acting on separate receptors, activate a common Ca^{++} influx site or channel (13, 19). The Ca-dependent phase of the K^+ release response was antagonized by a number of agents, regardless of the agonist employed. Cobalt was one such agent suggested to antagonize (perhaps competitively) the inward movement of Ca^{++} (13). Procaine, on the other hand, blocked Ca-dependent and Ca-independent phases of the K^+ release response when carbachol or phenylephrine was agonist but not when substance P was agonist (13). The data in Fig. 9 show that 3.0 mM $CoCl_2$ blocks Ca^{++} influx due to carbachol or substance P, while 1.0 mM procaine blocks influx due to carbachol but not substance P. These results confirm the contention that these agents act on the Ca^{++} influx mechanism rather than a subsequent step leading to K^+ release (13, 19). Since carbachol and substance P apparently activate the same Ca^{++} influx site, the differential sensitivity to procaine suggested that the local anesthetic might act to interrupt coupling between the muscarinic receptor and the Ca^{++} channel. The coupling mechanism for the substance P receptor was suggested to be insensitive to procaine for unknown reasons (13). While the results with Ca^{++} influx measurements obtained here provide substantial support for these contentions, they shed no light on the nature of the different coupling mechanisms themselves. Continued investigation may reveal the character of these processes and provide information on basic mechanisms of control of ion permeability by receptors.

In summary, this report suggests that stimulation of any of several receptors enhances uptake of ^{45}Ca by dispersed parotid acinar cells. In the case of *beta* adrenergic stimulation, the uptake may be (at least partially) due to pinocytosis. Carbachol and substance P probably stimulate transmembrane Ca^{++} influx. Cobalt can antagonize the inward movement of Ca^{++} due to carbachol or substance P, while procaine specifically antagonizes the cholinergic mechanism.

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