Adrenergic-agonist-induced Ca^{2+} fluxes in rat parotid cells are not Na^{+} -dependent

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We investigated the hypothesis that extracellular Na^{+} is required for the rapid mobilization of Ca^{2+} by rat parotid cells after adrenergic stimulation. When Na^{+} salts in the media were osmotically replaced with either choline chloride (+ atropine) or sucrose, efflux of $^{45}\text{Ca}^{2+}$ from preloaded cells, caused by $10\mu\text{M}$ (–)-adrenaline, was unchanged. Similarly adrenaline stimulated $^{45}\text{Ca}^{2+}$ uptake into cells under non-steady-state conditions in the presence or absence of Na^{+} . Monensin, a Na^{+} ionophore, was able to elicit a modest increase in $^{45}\text{Ca}^{2+}$ efflux, compared with controls. Studies of net $^{45}\text{Ca}^{2+}$ flux, performed under near-steady-state conditions, showed that adrenaline caused net $^{45}\text{Ca}^{2+}$ accumulation, whereas monensin caused net $^{45}\text{Ca}^{2+}$ release. The effect of monensin required the presence of Na^{+} in the incubation medium. Both 1mM - LaCl_3 and 0.1mM -D-600 prevented adrenaline-stimulated $^{45}\text{Ca}^{2+}$ uptake into cells, but had no effect on monensin-induced changes. We conclude that (1) the rapid mobilization of Ca^{2+} by adrenergic agonists seen in rat parotid cells does not require a $\text{Na}^{+}_{\text{out}} > \text{Na}^{+}_{\text{in}}$ gradient and (2) the nature of the monensin effect is quite different from the adrenergic-agonist-induced response.

Mobilization of intracellular Ca^{2+} is a key part of the mechanism by which several neurotransmitter receptors mediate their physiological effects (Putney, 1978a; Wollheim & Sharp, 1981). Among the many model systems available for studying such receptor coupling, frequently exocrine secretion from salivary glands is utilized and Ca^{2+} mobilization is measured as changes in $^{45}\text{Ca}^{2+}$ uptake, efflux or net flux. For example, in the rat parotid gland, stimulation at α_1 -adrenergic, muscarinic-cholinergic and substance-P-peptidergic receptors elicits secretion after release of Ca^{2+} from a common cellular pool (Marier *et al.*, 1978; Butcher, 1980; Poggioli & Putney, 1982).

Considerable effort has been directed at characterizing mechanistic features of these receptor-activation events (e.g. Putney, 1978b; Berridge *et al.*, 1982; Weiss *et al.*, 1982; Michell, 1983; Bodner *et al.*, 1983). Subsequent data suggest that Ca^{2+} mobilization is likely in response to the generation of inositol trisphosphate by cells after agonist

binding (Berridge, 1984). Exactly by what process(es) Ca^{2+} is mobilized, however, is not known.

Ca^{2+} movement across mammalian-cell plasma membranes involves distinct efflux and influx pathways. Extrusion of Ca^{2+} is most commonly accomplished by $\text{Na}^{+}/\text{Ca}^{2+}$ exchange or by an ATP-dependent process (Blaustein, 1974; Carafoli & Crompton, 1978; Wollheim & Sharp, 1981). Depending on the direction of the Na^{+} gradient (intracellular > or < extracellular), $\text{Na}^{+}/\text{Ca}^{2+}$ exchange could take up as well as extrude Ca^{2+} . Influx of Ca^{2+} occurs through several mechanisms. These include a basal 'leak' as well as potential-dependent and receptor-operated Ca^{2+} channels (Janis & Triggle, 1983).

It has been demonstrated (Putney & Parod, 1978; Landis & Putney, 1979) that, after activation of the α_1 -adrenergic, muscarinic-cholinergic and substance-P-peptidergic receptors, parotid cells show increased uptake of radiolabelled Na^{+} . This increased membrane permeability to Na^{+} is consistent with the transient hyperpolarization which accompanies neurotransmitter stimulation of parotid acinar cells (Pedersen & Petersen, 1973). Indeed, others have suggested that Ca^{2+} -mediated secretory responses from salivary glands may

Abbreviation used: HBSS, Hanks' balanced salt solution with 30mM-Hepes, pH 7.4.

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require Na^+ and that Na^+ may have a role in receptor-coupled Ca^{2+} mobilization (Keryer *et al.*, 1979; Watson *et al.*, 1981). We have previously demonstrated that parotid-cell basolateral membranes do possess a $\text{Na}^+/\text{Ca}^{2+}$ -exchange mechanism (Takuma *et al.*, 1985). However, a detailed assessment of Na^+ -dependence for parotid Ca^{2+} fluxes induced by neurotransmitter agonists has not been performed. In the present study we have addressed this issue and conclude that Na^+ is not required for the rapid Ca^{2+} mobilization seen with parotid cells after adrenergic stimulation.

Materials and methods

Materials

Animals were obtained from Harlan-Sprague-Dawley. Collagenase (type CLSPA) was from Worthington. $^{45}\text{CaCl}_2$ (2.2 mCi/ml) was from Amersham. (–)-Adrenaline, (±)-propranolol, hyaluronidase (type 1-S) and LaCl_3 were from Sigma. Monensin was purchased from Calbiochem. Methoxyverapamil (D-600) was given by Dr. Bruce Seligmann, National Institute of Allergy and Infectious Diseases, N.I.H. All other reagents were of the highest grade commercially available.

Cell preparation

For all studies, Wistar-strain rats (350–400 g) were utilized. Animals were killed between 9:30 and 10:30 h after diethyl ether anaesthesia. After excision and fine mincing of the parotid gland, cells were obtained essentially as described by Ito *et al.* (1981). Briefly tissue was incubated for 1 h in HBSS containing collagenase (96 units/ml) and hyaluronidase (0.19 mg/ml), gassed every 20 min with O_2/CO_2 (19:1) and subjected to gentle pipetting. Resulting cells were washed three times with HBSS containing 0.033% bovine serum albumin.

Uptake of $^{45}\text{Ca}^{2+}$

After the digestion, cells were placed in HBSS or in HBSS in which all NaCl was replaced osmotically by sucrose or by choline chloride with atropine (20 μM). Also Na_2HPO_4 was replaced by KH_2PO_4 in this modified medium so that the medium was free of all added Na^+ . The exact compositions of different media used here were as follows: HBSS, 137 mM- NaCl , 5.4 mM- KCl , 0.4 mM- KH_2PO_4 , 0.33 mM- Na_2HPO_4 , 0.81 mM- MgSO_4 , 1.27 mM- CaCl_2 , 5.5 mM-glucose, 30 mM-Hepes, pH 7.4; choline chloride medium, 139 mM-choline chloride, 5.4 mM- KCl , 0.8 mM- KH_2PO_4 , 0.81 mM- MgSO_4 , 1.27 mM- CaCl_2 , 5.5 mM-glucose, 30 mM-Hepes, pH 7.4; sucrose medium, 275 mM-sucrose, 5.4 mM- KCl , 0.8 mM- KH_2PO_4 , 0.8 mM- MgSO_4 , 1.27 mM- CaCl_2 , 5.5 mM-glucose, 30 mM-Hepes, pH 7.4. Cells

were then incubated with $^{45}\text{Ca}^{2+}$ (4 $\mu\text{Ci}/\text{ml}$) with or without different agonists and/or antagonists, for 15 min. Cells were centrifuged (750 g, 15 s), media were removed, and cells were rapidly washed with 4 mM-EGTA once, resuspended in phosphate-buffered saline (154 mM- $\text{NaCl}/10$ mM-sodium phosphate, pH 7.4) without Ca^{2+} and homogenized with a Polytron PT-10 for 10 s at setting 5. Samples of the homogenate were assayed for radioactivity and for DNA (Richards, 1974) and the results expressed as pmol of $^{45}\text{Ca}^{2+}$ taken up/ μg of DNA.

Net $^{45}\text{Ca}^{2+}$ flux

In two experiments, studies of net $^{45}\text{Ca}^{2+}$ flux were performed with a different experimental design (Poggioli & Putney, 1982). After digestion, cells were incubated for 1 h in HBSS with $^{45}\text{Ca}^{2+}$ to obtain near-steady-state amounts of tracer. The time course for $^{45}\text{Ca}^{2+}$ accumulation (not shown) was identical with that shown by Poggioli & Putney (1982). Thereafter various agents were added for a period of 15 min directly to the media. Cells were washed with 4 mM-EGTA once, resuspended in phosphate-buffered saline, and the amount of $^{45}\text{Ca}^{2+}/\mu\text{g}$ of DNA was determined as a measure of net Ca^{2+} flux.

Efflux of $^{45}\text{Ca}^{2+}$

After the digestion, the cells were incubated for 1 h in HBSS containing 4 μCi of $^{45}\text{Ca}^{2+}/\text{ml}$ as above. Thereafter cells were washed three times with HBSS and resuspended in HBSS in which the Na^+ was replaced (as above). Different adrenergic agents and/or the Na^+ ionophore monensin were added for 1 or 5 min. The cells were centrifuged (750 g, 15 s) and a portion of the medium was removed. The cells and remaining media were homogenized, and samples of the homogenate and the media were assayed for radioactivity. Release of $^{45}\text{Ca}^{2+}$ into the media was expressed as percentage of total $^{45}\text{Ca}^{2+}$ present.

Results

In all experiments (–)-adrenaline was utilized as an adrenergic agonist. At 10 μM -adrenaline, with these cells, maximum secretory responsiveness is observed (Bodner *et al.*, 1983). Typically, with incubations performed in HBSS, adrenaline gives a marked enhancement of $^{45}\text{Ca}^{2+}$ flux, as has been demonstrated by others (e.g. Koelz *et al.*, 1977; Miller & Nelson, 1977; Putney *et al.*, 1978; Landis & Putney, 1979). Thus adrenaline treatment for 1 min results in a 50–100% increase in $^{45}\text{Ca}^{2+}$ release from preloaded cells compared with results with control incubations (Fig. 1). Similarly, if uptake of $^{45}\text{Ca}^{2+}$ is measured for 15 min under non-steady-state conditions, adrenaline increased $^{45}\text{Ca}^{2+}$ influx by 20–50% (Fig. 2).

Fig. 1. Effect of replacement of Na^+ salts on $^{45}\text{Ca}^{2+}$ efflux. Rat parotid cells were pre-loaded with $^{45}\text{Ca}^{2+}$ for 60 min, washed and then incubated in paired experiments to determine the effect on $^{45}\text{Ca}^{2+}$ efflux of replacing NaCl osmotically in HBSS with either choline chloride containing $20\mu\text{M}$ -atropine (a) or sucrose (b). Media contained either $10\mu\text{M}$ -adrenaline + $50\mu\text{M}$ -propranolol (EPI) or $50\mu\text{M}$ -propranolol (CON). After 1 min incubation tubes were centrifuged, a sample of each medium was removed, and percentage $^{45}\text{Ca}^{2+}$ efflux calculated. Data are means $\pm$ S.E.M. for four (a) or five (b) paired experiments assayed in triplicate. Similar results were obtained if cells were incubated without propranolol (results not shown).

To test the possible requirement for Na^+ , in particular a $\text{Na}^+_{\text{out}} > \text{Na}^+_{\text{in}}$ gradient across the plasma membrane, in the mechanism of these adrenergic-agonist-induced $^{45}\text{Ca}^{2+}$ fluxes, we re-

moved all added Na^+ salts from the HBSS and replaced Na^+ salts with either choline chloride or sucrose. Incubations with choline chloride also included $20\mu\text{M}$ -atropine to block the muscarinic-agonist activity of the replacing cation. Therefore both types of replacement experiments retained the osmolarity of normal media, but 'sucrose-media' incubations also had diminished ionic strength.

Fig. 1 shows results of $^{45}\text{Ca}^{2+}$ -efflux experiments. For all studies presented herein, agonist incubations in HBSS were performed simultaneously with experimental media treatments, i.e. all cells were from the same preparations and therefore data are of paired studies. Net $^{45}\text{Ca}^{2+}$ release, 1 min after adrenergic-agonist addition, was essentially identical with or without Na^+ in the incubation media. With choline chloride present, basal percentage $^{45}\text{Ca}^{2+}$ release (12.4 ± 0.7 ; mean $\pm$ S.E.M., $n = 4$) was comparable with that in HBSS (12.9 ± 0.1). After stimulation, $^{45}\text{Ca}^{2+}$ efflux increased by approx. 55% under both conditions. Cells incubated with sucrose media showed consistently higher basal $^{45}\text{Ca}^{2+}$ release than for those in HBSS (12.8 ± 0.9 and 10.1 ± 1.0 respectively; $n = 5$, $t = 7.51$, $P = 0.0017$). Although net percentage efflux was the same (approx. 8%), the relative increment with sucrose-containing incubations was smaller (68% versus 85% increased over basal).

Similar experiments to evaluate $^{45}\text{Ca}^{2+}$ uptake were next performed, and these findings are shown in Fig. 2. When media containing choline chloride, plus atropine, were used, cells responded to adrenaline addition in similar fashion to cell response in HBSS. Under both situations $^{45}\text{Ca}^{2+}$ influx was significantly increased (by approx. 43%) over basal values during the 15 min test period. However, unstimulated uptake showed a consistent modest alteration, based on the content of the incubation medium. In all four experiments cells incubated with choline chloride displayed higher basal uptake ($86.4 \pm 1.2 \text{ pmol}/\mu\text{g}$ of DNA) than did cells in HBSS (73.6 ± 2.1). This difference was statistically significant ($t = 6.15$, $P = 0.009$). If sucrose was used to replace Na^+ salts, cells also were capable of responding to adrenaline treatment. However, in these paired experiments ($n = 5$) there was considerable difference between conditions in basal influx values, and in the percentage increase above basal influx stimulated by adrenaline (Fig. 2). Basal $^{45}\text{Ca}^{2+}$ uptake was increased by approx. 70% with sucrose media ($210.0 \pm 7.8 \text{ pmol}/\mu\text{g}$ of DNA) compared with that in HBSS (125.4 ± 16.3 ; $t = 7.72$, $P = 0.0015$). Also, in these experiments adrenaline elevated $^{45}\text{Ca}^{2+}$ influx by approx. 20% ($150.4 \pm 19.6 \text{ pmol}/\mu\text{g}$ of DNA) in control incubations, but consistently enhanced influx by approx. 60% (to 342.3 ± 23.8)

Fig. 2. Effect of replacement of Na^+ salts on $^{45}\text{Ca}^{2+}$ uptake

Rat parotid cells were incubated with $^{45}\text{Ca}^{2+}$ to determine the effect on $^{45}\text{Ca}^{2+}$ uptake of replacing NaCl osmotically in HBSS with either choline chloride containing $20\mu\text{M}$ -atropine (a) or sucrose (b). Paired experiments (comparing HBSS with modified medium) were performed. Media contained either $10\mu\text{M}$ -adrenaline (EPI) or buffer alone (CON). After 15 min cells were centrifuged, media removed, and cells washed and then homogenized. Samples of the homogenate were assayed for radioactivity and DNA. Data are means $\pm$ S.E.M. for four (a) or five (b) paired experiments assayed in triplicate.

over basal for cells incubated in sucrose media. This difference was statistically significant ($t = 6.64$, $P = 0.0027$).

Another approach that has been utilized to suggest a role for Na^+ in physiological processes is to see if the Na^+ ionophore monensin can mimic the event being studied (see, e.g., Watson *et al.*, 1981; DiVirgilio & Gomperts, 1983). Accordingly cells were incubated, in parallel, in HBSS and

either adrenaline or monensin ($50\mu\text{M}$, chosen on the basis of the data of Watson *et al.*, 1981), and $^{45}\text{Ca}^{2+}$ fluxes were evaluated. Monensin was dissolved in ethanol, and thus all control incubations for the Na^+ ionophore included 1% (v/v, final concn.) ethanol in the media. Ethanol addition alone had no significant effect on $^{45}\text{Ca}^{2+}$ fluxes (Figs. 3 and 4).

In the first series of ionophore studies $^{45}\text{Ca}^{2+}$ efflux was evaluated after 1 min exposure to test agents (Fig. 3). Adrenaline caused a 92% increase in percentage efflux (18.8 ± 1.9 versus 9.8 ± 1.3 , $n = 4$) in these experiments, whereas monensin elicited a much smaller response (approx. 30%; 11.7 ± 0.5 versus 9.0 ± 0.2). This difference in responsiveness was significant ($t = 17.3$, $P = 0.001$). To ascertain if these agents mobilized the same or different $^{45}\text{Ca}^{2+}$ pools, cells were incubated with both agents, and efflux responses compared with those obtained in the presence of only one compound. As shown in Table 1, there was no additive response when $^{45}\text{Ca}^{2+}$ efflux was measured in cells treated with both adrenaline and monensin. Earlier work by Watson *et al.* (1981) had shown a large monensin-induced $^{45}\text{Ca}^{2+}$ release from mouse parotid cells after 5 min incubation. We therefore repeated our studies at this later time point. These data are also shown in Fig. 3. Once again adrenaline treatment resulted in a much greater $^{45}\text{Ca}^{2+}$ efflux (162% over basal) than did monensin treatment (82% above basal), ($t = 5.7$, $P = 0.011$).

Next we evaluated the ability of monensin to affect $^{45}\text{Ca}^{2+}$ uptake under non-steady-state conditions. These results are shown in Fig. 4. Cells incubated with adrenaline displayed approx. 44% more $^{45}\text{Ca}^{2+}$ uptake than did untreated control cells (134.6 ± 9.7 versus 93.7 ± 6.6 pmol/ μg of DNA; $n = 4$, $t = 4.12$, $P = 0.026$). Conversely, in paired experiments with monensin, cells incubated with the Na^+ ionophore took up significantly less $^{45}\text{Ca}^{2+}$ (approx. 17%) than did their untreated controls (87.3 ± 4.6 versus 104.8 ± 6.3 ; $n = 4$, $t = 3.75$, $P = 0.033$). To be sure that this result was not a non-specific action of monensin on parotid cells, but rather was related to its ionophoric properties, we attempted to assess if this response required Na^+ . As shown in Table 2, although monensin significantly decreased $^{45}\text{Ca}^{2+}$ uptake when cells were incubated in HBSS ($t = 5.02$, $P = 0.037$), the ionophore had little effect when the same cells were incubated in HBSS containing choline chloride. Thus this monensin response is Na^+ -dependent.

These results strongly suggested considerable differences in the Ca^{2+} -mobilization responses of adrenergic agents and monensin. To assess this more carefully, we evaluated the effects of monen-

Fig. 3. Effect of adrenaline and monensin on $^{45}\text{Ca}^{2+}$ efflux from parotid cells

Cells were pre-loaded with $^{45}\text{Ca}^{2+}$ for 60 min, washed and then incubated in HBSS with $1\text{ }\mu\text{M}$ -adrenaline (E), $50\text{ }\mu\text{M}$ -monensin (M), medium alone (C) or medium containing 1% (v/v) ethanol (C'). $^{45}\text{Ca}^{2+}$ efflux was assessed after 1 min (left) or 5 min (right) as in Fig. 1. Data are means $\pm$ S.E.M. for four paired experiments assayed in triplicate.

Table 1. Effect of adrenaline and monensin, added simultaneously, on $^{45}\text{Ca}^{2+}$ efflux

Rat parotid cells were pre-loaded with $^{45}\text{Ca}^{2+}$ for 60 min, washed and then incubated in HBSS with adrenaline, or monensin, or both, at the concentrations indicated. $^{45}\text{Ca}^{2+}$ efflux was assessed after 1 min. Data are averages of two experiments assayed in triplicate. Individual experimental values were within $\pm 5\%$ of average values.

Treatment	Efflux (%)	Change from control efflux (%)
Control	10.9	—
Adrenaline ($10\text{ }\mu\text{M}$)	17.0	+56
Monensin ($50\text{ }\mu\text{M}$)	12.7	+16.5
Adrenaline ($10\text{ }\mu\text{M}$) + monensin ($50\text{ }\mu\text{M}$)	16.7	+53

sin and of adrenaline on net $^{45}\text{Ca}^{2+}$ fluxes, when $^{45}\text{Ca}^{2+}$ was at near-steady-state conditions as described by Poggioli & Putney (1982). These results are shown in Table 3. Clearly, as others have shown (Poggioli & Putney, 1982), the net

effect of treating rat parotid cells with an adrenergic agonist is an increase in cellular $^{45}\text{Ca}^{2+}$ (approx. 13% increase over control untreated cells). In marked contrast with this, monensin treatment, and the resulting dissipation of the transmembrane Na^+ gradient, yields a net decrease of cellular $^{45}\text{Ca}^{2+}$ (approx. 13%).

We next attempted several experimental manipulations to characterize better the nature of the adrenergic and monensin effects. Firstly, incubation media were depleted of Ca^{2+} (no added Ca^{2+} to the HBSS + inclusion of 1 mM -EGTA), and $^{45}\text{Ca}^{2+}$ efflux induced by adrenaline and monensin was measured. Both agents showed accelerated efflux when EGTA was present, but, as described above, the adrenaline-induced efflux was much greater than that with monensin (results not shown). This increased adrenergic-induced efflux with Ca^{2+} -depleted media is similar to results reported by others (Miller & Nelson, 1977; Poggioli & Putney, 1982). Secondly, we assessed the ability of both an inorganic (LaCl_3) and an organic (D-600) Ca^{2+} -channel blocker to affect the adrenaline- and monensin-induced $^{45}\text{Ca}^{2+}$ -flux

Fig. 4. Effect of adrenaline and monensin on $^{45}\text{Ca}^{2+}$ uptake in parotid cells

Cells were incubated with $^{45}\text{Ca}^{2+}$ in HBSS containing $10\text{ }\mu\text{M}$ -adrenaline (E), $50\text{ }\mu\text{M}$ -monensin (M), medium alone (C) or medium containing ethanol (final concn. 1% , v/v) (C'). After 15 min cells were centrifuged, media removed, and cells washed and then homogenized. Samples of the homogenate were assayed for radioactivity and DNA. Data are means $\pm$ S.E.M. for four paired experiments assayed in triplicate.

patterns. The results are shown in Table 4. Both types of Ca^{2+} -channel blockers prevented the increased $^{45}\text{Ca}^{2+}$ uptake usually seen after adrenaline treatment of parotid cells (Figs. 2 and 4). Indeed, on average, adrenaline-treated cells showed slightly less $^{45}\text{Ca}^{2+}$ uptake than did their respective controls. Conversely the Ca^{2+} -channel blockers appeared to have no effect on the monensin response (cf. Table 2 and Fig. 4).

Discussion

It was the purpose of the present investigation to assess the hypothesis that adrenergic activation of Ca^{2+} flux in rat parotid cells was Na^{+} -dependent. Previously, others have suggested that Na^{+} has a key role in Ca^{2+} -coupled autonomic-receptor-regulated parotid exocrine secretory events

Table 2. Monensin-induced changes in $^{45}\text{Ca}^{2+}$ fluxes are Na^{+} -dependent

Rat parotid cells were incubated with $^{45}\text{Ca}^{2+}$ in HBSS or media in which Na^{+} salts were replaced osmotically with choline chloride containing $20\text{ }\mu\text{M}$ -atropine. Cells were incubated in parallel for 15 min in the absence (control) or presence of $50\text{ }\mu\text{M}$ -monensin. Thereafter cells were centrifuged, washed and then homogenized. Samples of the homogenate were assayed for radioactivity and DNA. Data are presented as pmol of $^{45}\text{Ca}^{2+}$ / μg of DNA and are means $\pm$ S.E.M. for three paired experiments assayed in triplicate.

Treatment	$^{45}\text{Ca}^{2+}$ in cells	Change from control (%)
(1) HBSS		
Control	171.6 ± 8.0	—
Monensin	140.0 ± 9.7	−18.4
(2) Choline chloride		
Control	179.8 ± 7.1	—
Monensin	172.4 ± 9.2	−4.1

Table 3. Effect of adrenaline and monensin on net $^{45}\text{Ca}^{2+}$ fluxes

Rat parotid cells were incubated with $^{45}\text{Ca}^{2+}$ for 60 min to achieve near-steady-state conditions (Poggioli & Putney, 1982). Thereafter experimental agents were added directly to the media. Control incubations were performed with HBSS, and Control' included 1% (v/v) ethanol. Adrenaline and monensin were added at the indicated concentrations. Data are presented as pmol of $^{45}\text{Ca}^{2+}$ / μg of DNA, and are averages of two experiments assayed in triplicate. Individual values were within $\pm 5\%$ of the average values.

Treatment	$^{45}\text{Ca}^{2+}$ in cells	Change from control (%)
Control	96.2	—
Adrenaline ($10\text{ }\mu\text{M}$)	108.4	+12.7
Control'	105.8	—
Monensin ($50\text{ }\mu\text{M}$)	91.4	−13.6

(Keryer *et al.*, 1979; Watson *et al.*, 1981). Also, Na^{+} -dependent autonomic-receptor mobilization of intracellular Ca^{2+} has been suggested in other cells (see, e.g., Williams, 1975; Suchard *et al.*, 1982; Connolly *et al.*, 1984). It is clear, however, from the present work that the early Ca^{2+} fluxes initiated by adrenergic-receptor stimulation do not require extracellular Na^{+} , i.e. a $\text{Na}^{+}_{\text{out}} > \text{Na}^{+}_{\text{in}}$ trans-plasma-membrane gradient. Replacement of Na^{+} salts in HBSS with choline chloride, plus atropine, resulted in essentially no alteration in the response of cells to adrenaline. Replacement of Na^{+} salts by

Table 4. *Effect of Ca^{2+} -channel blockers on $^{45}\text{Ca}^{2+}$ uptake in the presence of adrenaline or monensin*

Rat parotid cells were incubated with $^{45}\text{Ca}^{2+}$ in HBSS, to which 10 mM- LaCl_3 (Expt. 1) or 0.1 mM-D-600 (Expt. 2) were added. Cells were incubated in parallel for 15 min in the absence (control, either LaCl_3 or D-600 alone) or presence of either 10 μM -adrenaline or 50 μM -monensin. Thereafter cells were centrifuged, washed and then homogenized. Samples of the homogenate were assayed for radioactivity and DNA. Data are presented as pmol of $^{45}\text{Ca}^{2+}/\mu\text{g}$ of DNA, and for Expt. 1 are means $\pm$ S.E.M. for three studies, and for Expt. 2 the mean of one study, all assayed in triplicate.

Expt. no.	Treatment	$^{45}\text{Ca}^{2+}$ in cells	Change from control (%)
1	Control	219.3 $\pm$ 35.4	—
	Adrenaline	189.5 $\pm$ 50.2	-13.6
	Control	207.7 $\pm$ 33.6	—
	Monensin	164.2 $\pm$ 35.7	-20.9
2	Control	51.8	—
	Adrenaline	47.5	-8.3
	Control	62.3	—
	Monensin	32.0	-48.6

sucrose also allowed for the adrenergic stimulation of $^{45}\text{Ca}^{2+}$ fluxes. However, there was some quantitative difference in cellular responsiveness to adrenaline in sucrose media compared with normal HBSS. It is likely that this is due in part to the marked decrease in the ionic strength of the incubation media and also to the lack of Cl^- present. As noted by Putney (1978b), this is likely to produce a depolarization response itself and consequently to alter Ca^{2+} fluxes.

As an additional test of a role for Na^+ in adrenergic regulation of parotid-cell Ca^{2+} fluxes, we evaluated the ability of monensin, a Na^+ ionophore, to mimic the adrenaline response. Monensin treatment would rapidly increase plasma-membrane permeability to Na^+ , and soon dissipate the pre-existing Na^+ gradient. Our results show that, although monensin can elicit $^{45}\text{Ca}^{2+}$ efflux from parotid cells, (1) it is significantly lower than that with adrenaline, and (2) most importantly, it is not indicative of the same pattern of net Ca^{2+} flux which results from stimulation by the adrenergic agonist. Although additivity experiments (Table 1) suggest that the ionophore and the agonist may affect a similar Ca^{2+} pool, net-flux studies show that monensin does not in fact mimic the adrenergic response.

We attempted to address the issue of why these responses are different first by experiments employing Ca^{2+} -depleted media and second by using Ca^{2+} -channel blockers. The former approach was

not useful in distinguishing between the response types, but the latter was. Both LaCl_3 and D-600 prevented the adrenaline-induced increase in $^{45}\text{Ca}^{2+}$ uptake, but were without effect on the monensin response. Inorganic and organic Ca^{2+} -channel blockers inhibit Ca^{2+} influx by acting at apparently different membrane cation-binding sites (Lee & Tsien, 1983). Although studies with organic blockers are complicated by the fact that these compounds can also function as α -adrenergic antagonists (Atlas & Adler, 1981; Frelin *et al.*, 1982), our results with both classes of agents are consistent with the findings of Marier *et al.* (1978) that Ca^{2+} -dependent K^+ release from rat parotid cells is blocked by LaCl_3 and D-600. Apparently monensin simply mobilizes cellular Ca^{2+} by a Na^+ -exchange mechanism (Table 2) from a pool that is also a target of adrenergic agonists (Table 1). Adrenaline can mobilize not only the monensin-target Ca^{2+} pool but probably other cellular pools. In support of this, adrenergic-induced $^{45}\text{Ca}^{2+}$ efflux is substantially greater than monensin-induced efflux (Table 1, Fig. 3). Also a major target Ca^{2+} pool, in exocrine cells, for neurotransmitters is the endoplasmic reticulum (Streb *et al.*, 1984). Apparently Ca^{2+} flux in this pool is not Na^+ -dependent (Case, 1984). Furthermore, adrenaline stimulates Ca^{2+} uptake, which monensin cannot do. Thus, not surprisingly, it seems that the adrenergic response is kinetically more complicated than that of the ionophore and results in a different direction of net Ca^{2+} movement.

Although these studies argue convincingly that rapid Ca^{2+} mobilization by adrenergic agonists is not Na^+ -dependent, they do not exclude the possibility that Na^+ is important to secretory events related to parotid-cell Ca^{2+} handling. For example, we have studied Ca^{2+} -transport mechanisms in parotid basolateral-membrane vesicles, and found that Na^+ can significantly inhibit ATP-dependent Ca^{2+} extrusion across this membrane (Takuma *et al.*, 1985). Since adrenergic stimulation results in enhanced $^{22}\text{Na}^+$ uptake by parotid cells (Landis & Putney, 1979), it seems reasonable to suggest that elevated intracellular Na^+ uptake might inhibit ATP-dependent Ca^{2+} extrusion, thereby prolonging increased free Ca^{2+} within the cell and sustaining a secretory response.

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