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Cellular responses to Ca^{2+} from extracellular and intracellular sources are different as shown by simultaneous measurements of cytosolic Ca^{2+} and secretion from bovine chromaffin cells

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ABSTRACT **Bovine adrenal medullary cells, cultured on quartz plates, were superfused with buffer to which pulses of stimulant were added. Cytosolic Ca^{2+} was measured by the fura-2 fluorescence method and the simultaneously released catecholamine was measured electrochemically. When stimulant concentrations were adjusted to given equivalent elevations of cytosolic Ca^{2+}, secretion depended entirely on whether Ca^{2+} came from internal stores or from the extracellular medium. Calcium from internal stores did not support secretion under these conditions. This nonequivalence of the two sources of cytosolic Ca^{2+} points to important differences in the physiological roles of the two sources of calcium. Dimethylphenylpiperazinium (a cholinergic agonist) and elevated K^+ increased cytosolic Ca^{2+} and caused secretion only in the presence of external Ca^{2+}. Bradykinin, muscarine, and ATP elevated cytosolic Ca^{2+} in the presence and absence of extracellular Ca^{2+} but caused secretion only in the presence of extracellular Ca^{2+}. UTP, which in the absence of extracellular Ca^{2+} elevated cytosolic Ca^{2+} as effectively as ATP, did not cause detectable secretion under any circumstance. Because of the high Ca^{2+}-buffering capacity of the cytosol, we expected that Ca^{2+} gradients, perhaps quite steep, would be produced by a pulse of Ca^{2+} entering the cytosol. Fura-2 fluorescence measures only the average free cytosolic Ca^{2+}. Our data show that Ca^{2+} entering across the plasma membrane was much more effective at triggering exocytosis than was Ca^{2+} released from internal stores, suggesting that the two sources of Ca^{2+} are effectively compartmentalized, probably by concentration gradients in the cytosol.**

Douglas (1) demonstrated that entry of Ca^{2+} into cells of the adrenal medulla is necessary and sufficient to induce secretion of catecholamine. Stimulation by acetylcholine, by depolarizing concentrations of K^+ in the medium, or by Ca^{2+} entry produced by ionophores requires the presence of Ca^{2+} in the extracellular medium. Subsequent experiments have generally confirmed that hypothesis and extended it to cover all cells that secrete by exocytosis: endocrine cells, neuronal cells, and others, such as salivary gland cells and mast cells. Adrenal cells permeabilized by electric fields or detergents require Ca^{2+} for exocytotic secretion (2–4).

It has become apparent that secretory cells have substantial internal stores of Ca^{2+} and that this Ca^{2+} can be released into the cytoplasm upon stimulation of cell surface receptors coupled to phospholipase C. Hydrolysis of phosphatidylinositol bisphosphate by phospholipase C releases inositol trisphosphate, which is the second messenger that releases Ca^{2+} from the intracellular stores (5).

The calcium released from internal stores can reach substantial concentrations in the cytoplasm and can presumably activate many Ca^{2+}-dependent processes related to signal transduction, such as activation of phospholipase C, calmodulin-dependent cyclic nucleotide phosphodiesterase, protein kinase, and protein phosphatase. Combined measurements of cytosolic Ca^{2+} and secretion now allow us to compare secretion evoked by Ca^{2+} from either source at equivalent levels of cytosolic Ca^{2+}. Our evidence shows that Ca^{2+} released from internal stores does not induce exocytosis from these cells.

MATERIALS AND METHODS

Cell Culture. Chromaffin cells were isolated from bovine adrenal medulla by collagenase digestions and purified by centrifugation on a Percoll gradient (6). The cells were cultured on quartz plates (1.25 × 4.5 cm), about 1 mm thick, that had been treated with fibronectin to promote cell adhesion. The cells were maintained up to 7 days in Eagle's minimal essential medium (GIBCO) containing 10% (vol/vol) heat-inactivated fetal calf serum (HyClone) plus antibiotics.

Measurement of Fura-2 Fluorescence and Catecholamine Release. Fura-2 fluorescence was used to measure cytosolic calcium (7). Chromaffin cells attached to quartz plates were incubated with 2–3 μM fura 2 tetrakis(acetoxymethyl) ester in Eagle's minimal essential medium at 37°C for 45–60 min. After incubation, cells were washed three times with Lockes solution (154 mM NaCl/5.6 mM KCl/2.2 mM $CaCl_2$/10 mM glucose/5 mM Hepes, pH 7.3) before being placed in a flow chamber. The quartz plate with attached cells formed one side of the flow chamber of $\approx$40 μl that was placed in the excitation beam of a Perkin–Elmer fluorescence spectrophotometer at an angle of 32° to the incident beam. Lockes solution flowed over the cells at a rate of 1 ml/min, at 29°C. The effluent from this flow chamber, in which fluorescence was measured, went immediately through capillary tubing to a Metrohm electrochemical detector to measure catecholamine released from the cells. In all experiments presented here, 6-sec pulses of the nicotinic agonist, 1,1-dimethyl-4-phenylpiperazinium (DMPP), elevated K^+ concentration, or other stimulants were injected into the flowing solution by a manual HPLC injection valve. There was a delay of 17 sec from the flow cell to the electrochemical detector. In the figures shown, the catecholamine curves have been made coincident with the fura-2 curve by using the known time delay factor. The dispersion ratios from the injection valve to the flow chamber and to the electrochemical detector are 0.56 and 0.37, respectively. In experiments not shown, we used digital imaging techniques to examine fura-2 fluorescence in individual cells. In no case did we see punctate or inhomogenious cytosolic fluorescence. The fura-2 seems to be uniformly distributed throughout the cytoplasm and not concentrated in vesicles of any type. Examination of individual cells also showed unequivocally that a low percentage (5–

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Abbreviation: DMPP, 1,1-dimethyl-4-phenylpiperazinium.

8%) of contaminating cells did not contribute significantly to the measured fluorescence.

Calibration of Ca^{2+} and Catecholamine Concentration. For these experiments, the increase of cytosolic Ca^{2+} was measured as an increase in fluorescence during excitation at 340 nm. Before and after stimulation, measurements of fluorescence were made with excitation at 360 nm, the isosbestic point of the absorption curves for free and Ca^{2+}-bound fura-2. During all measurements reported, the fluorescence caused by excitation at 360 nm did not change. This shows that the instrument was stable and that fura-2 did not bleach or leak from the cells during the experiment. Calibration of the fluorescence signal to intracellular Ca^{2+} concentration was made by standard procedures (7) but, since these calibrations are not absolute (8–10), we have usually shown results as fluorescence readings. In these experiments, the baseline Ca^{2+} concentrations were calculated to be near 150 nM and to increase up to 500 nM. The total catecholamine in cells in the chamber was ≈0.13 μmol, determined after trichloroacetic acid extraction. The highest level of secretion shown is ≈2% of the total cellular catecholamine. Responses of various cell batches differed from each other, but relative behavior with different stimulants was reproducible. All experiments have been carried out at least four times with consistent results.

RESULTS

We have measured the increase in cytosolic Ca^{2+} and concomitant release of catecholamine in response to short pulses of a variety of stimulants: DMPP (a nicotinic agonist), muscarine, bradykinin, ATP, UTP, and depolarizing concentrations of K^+. In Fig. 1, we show the transients in cytosolic Ca^{2+} concentration that resulted from K^+ or ATP stimulation of bovine chromaffin cells in the presence and absence of external Ca^{2+}. The resulting release of catecholamine is shown immediately above the fura-2 fluorescence signal. In the presence of 2.2 mM external Ca^{2+}, fura-2 signals showed that 50 μM ATP provoked a greater increase in internal Ca^{2+} than did 40 μM K^+. The release of catecholamine caused by the pulse of K^+, however, was ≈7 times greater than that caused by the pulse of ATP. The traces obtained in the absence of external Ca^{2+} show that, as expected, K^+-induced increases in cytosolic Ca^{2+} were entirely dependent on the extracellular source. The Ca^{2+} increase due to ATP was predominantly from internal stores, but there was a somewhat greater increase in cytosolic Ca^{2+} when extracellular Ca^{2+} was present than when it was not. The small amount of secretion triggered by ATP was eliminated when external Ca^{2+} was removed. Note that the rise in cytosolic Ca^{2+} caused by ATP in the absence of external Ca^{2+} is still appreciably greater than that caused by K^+ in the presence of external Ca^{2+}. The last trace of Fig. 1 shows that ATP was unable to induce secretion in the absence of external Ca^{2+} even if the cells were simultaneously depolarized. ATP and K^+ added together still did not cause secretion in the absence of external Ca^{2+}. Fig. 2 shows a similar set of experiments in which UTP was substituted for ATP. We found that UTP was the only other nucleoside triphosphate that mimiced ATP in releasing Ca^{2+} from internal stores. UTP, unlike ATP, triggered no catecholamine release, even in the presence of external Ca^{2+}. Again, the increase in cytosolic Ca^{2+} due to UTP could be substantially higher than that caused by K^+ concentrations that triggered substantial catecholamine release. In Fig. 3 it can be seen that when UTP and K^+ were given simultaneously, the Ca^{2+} transients appeared to be additive. However, the secretion was just that caused by elevated K^+ alone. As shown by traces D in Fig. 3, the secretion was not limited by saturation with Ca^{2+}. An increase of 50% in K^+ concentration caused an apparently small increase in cytosolic Ca^{2+} accompanied by a 4-fold increase in catecholamine secretion.

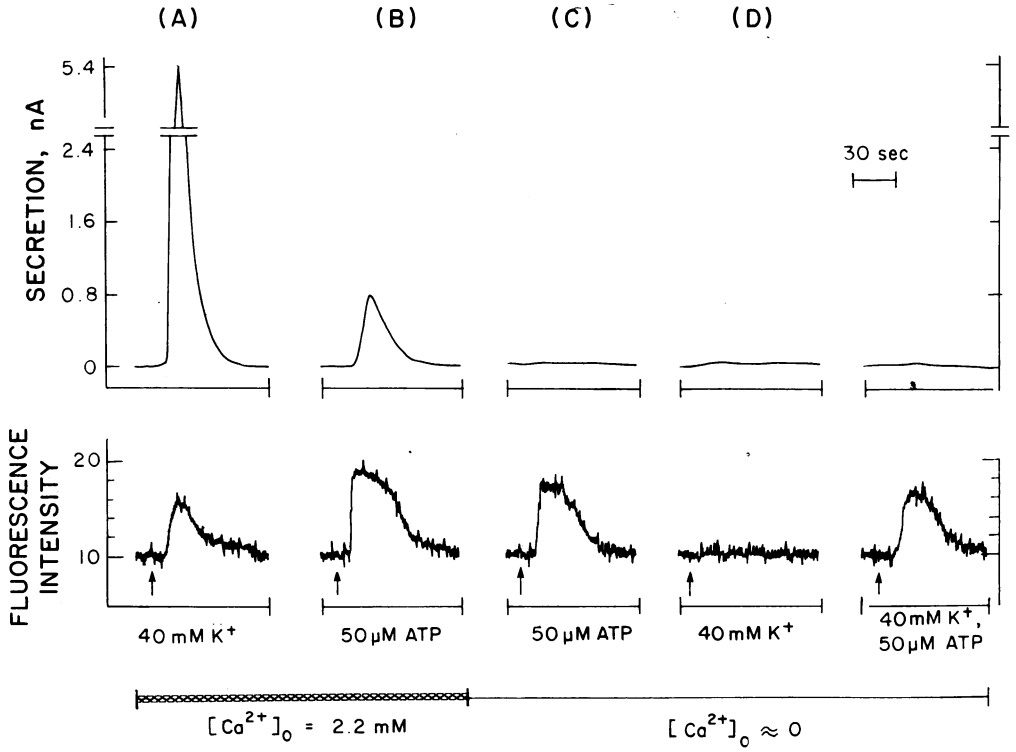

FIG. 1. Dependence of exocytosis on external calcium with ATP and K^+ as stimulants. The chromaffin cells were stimulated for 6 sec with 40 mM K^+ (traces A) and 50 μM ATP (traces B) in the presence of external calcium (2.2 mM) and with 50 μM ATP (traces C), 40 mM K^+ (traces D), and 40 mM K^+ plus 50 μM ATP (traces E) in the absence of extracellular calcium. (*Upper*) Release of catecholamines, determined electrochemically. (*Lower*) Fluorescence emission due to the fura 2–Ca^{2+} complex.

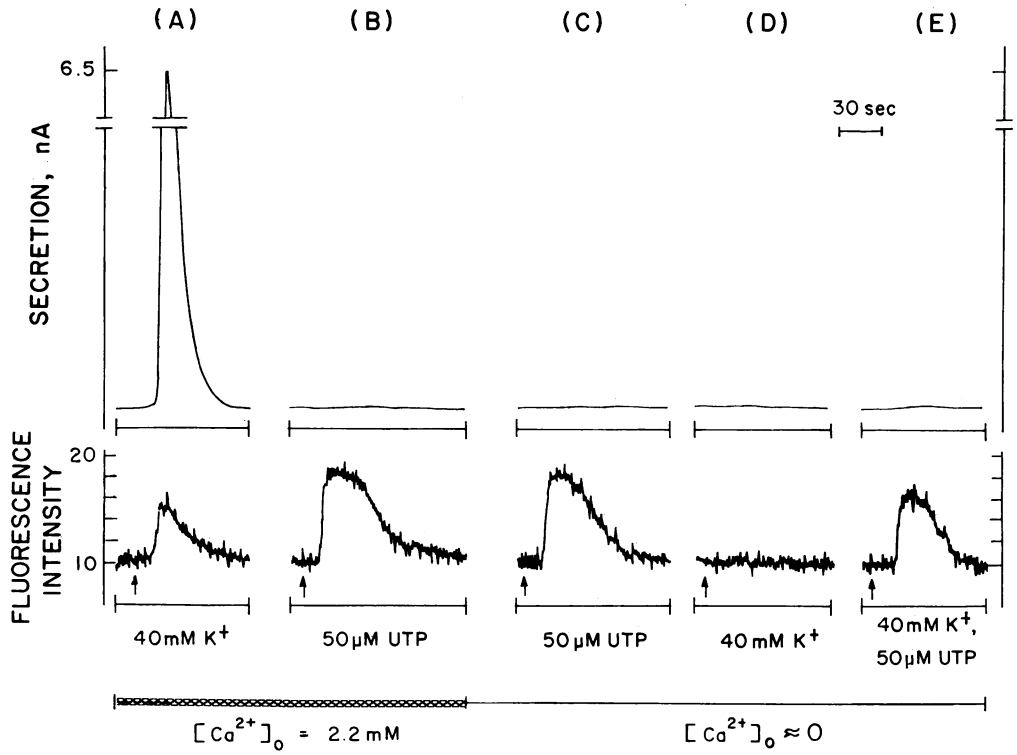

FIG. 2. Dependence of exocytosis on external Ca^{2+} with UTP and K^+ as stimulants. As in Fig. 1, but with stimulations by 40 mM K^+ (traces A) and 50 μM UTP (traces B) in the presence of external calcium and with 50 μM UTP (traces C), 40 mM K^+ (traces D), and 40 mM K^+ plus 50 μM UTP (traces E) in the absence of external calcium.

We have carried out several experiments using 5 μM DMPP instead of K^+. The results are the same as the results with K^+ in Figs. 1 and 2; DMPP triggered no increase in cytosolic Ca^{2+} in the absence of extracellular Ca^{2+}.

A pulse of 5 μM DMPP stimulated twice as much catecholamine release as 40 mM K^+ did but the increase in cytosolic Ca^{2+} was only half as great as that produced by 50 μM ATP or UTP.

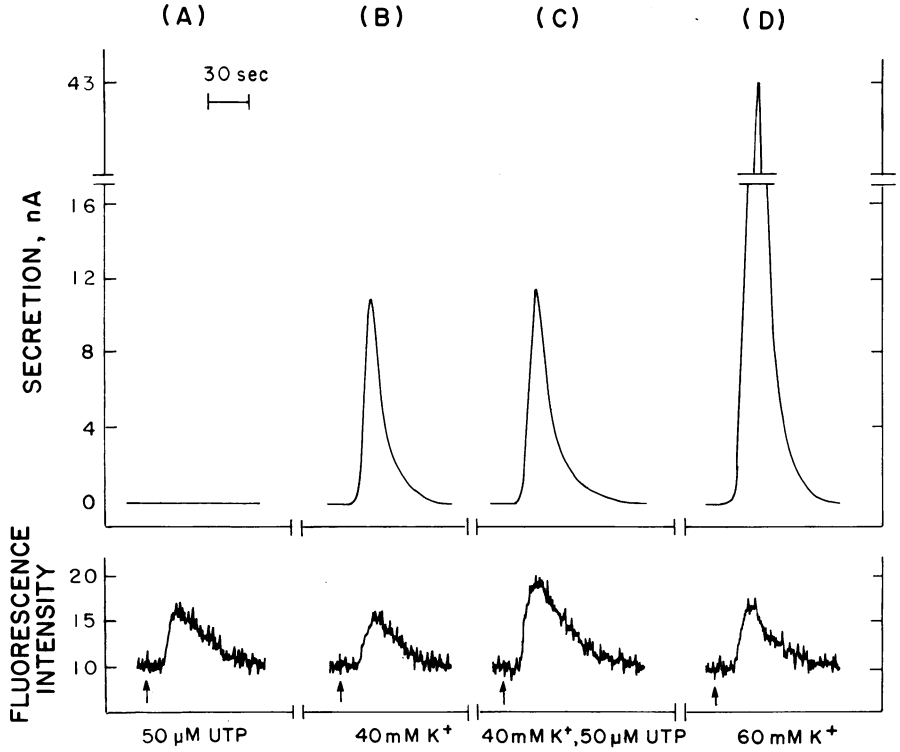

FIG. 3. UTP and K^+ additively enhance the calcium signal but not secretion. The chromaffin cells in the presence of 2.2 mM Ca^{2+} were exposed for 6 sec to 50 μM UTP (traces A), 40 mM K^+ (traces B), 40 mM K^+ plus 50 μM UTP (traces C), and 56 mM K^+ (traces D) to measure the internal free calcium increase and catecholamine secretion.

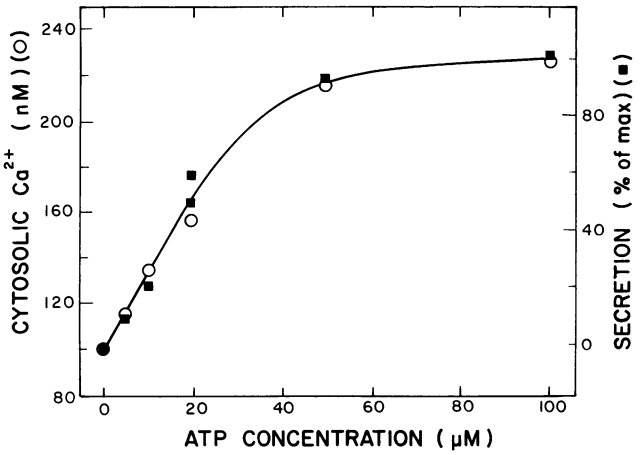

FIG. 4. Response of secretion and internal Ca^{2+} to ATP in the presence of 2.2 mM external Ca^{2+}. Six-second pulses of ATP (or UTP) were given 5 min apart to trigger responses similar to those in previous figures. ■, Relative amount of catecholamine secreted; ○, cytosolic Ca^{2+} concentrations calculated from fura-2 fluorescence.

Fig. 4 shows how secretion and the Ca^{2+} transient depend on ATP concentration. The half-maximal effect of ATP on secretion was between 10 and 20 μM. (Because of the dispersion factor, the peak ATP concentration at the cells was only 56% of the injected concentrations.) In Fig. 4 we have calculated calcium concentration from the fura fluorescence. Although there is uncertainty in the actual concentration values, the relative changes in concentration are more reliable.

In experiments not shown, the effects of UTP and ATP on the Ca^{2+} transient were compared at 10 μM, 50 μM, and 100 μM. In each case the increase caused by UTP was just slightly less than that caused by ATP, demonstrating a similar dose–response curve.

We have shown (unpublished data) that inositol 1,4,5-trisphosphate was generated to approximately the same extent when cells were exposed to either ATP or UTP, as expected from the release of internal Ca^{2+}. Bradykinin and muscarine are widely used agonists with receptors coupled to release of inositol trisphosphate into the cytosol (11). Data in Fig. 5 show that in bovine chromaffin cells both of these agonists did increase cytosolic Ca^{2+}. In the presence of external Ca^{2+}, both induced secretion. Neither produced secretion in the absence of external Ca^{2+} despite substantial elevation of cytosolic Ca^{2+}. Other experiments have shown that ATP was as effective at releasing Ca^{2+} from internal stores as were the better known agonists bradykinin and muscarine and that at saturating concentrations the effect of ATP was approximately additive with those of the other agonists. In our cells, the ATP, UTP, and bradykinin responses were highly reproducible; muscarinic responses were variable. We have had some cell batches in which elevation of cytosolic Ca^{2+} was 30–40% greater with muscarine than with ATP and other cell batches in which the response to muscarine was much lower or nonexistent. During culture of chromaffin cells there is a functional shift from muscarinic to nicotinic response (12). Quite likely, the rate and extent of this change will vary with culture conditions.

DISCUSSION

In this report we compare the effects of three classes of stimulants on cytosolic calcium concentration measured by the fura-2 method and on catecholamine secretion. The nicotinic agonist DMPP and elevated K^+ can elevate cytosolic Ca^{2+} only by inducing Ca^{2+} entry through the plasma membrane and cause catecholamine secretion only in the presence of external Ca^{2+}. Muscarine, bradykinin, and ATP elevate cytosolic Ca^{2+} chiefly from internal stores but they do not stimulate catecholamine secretion in the absence of external Ca^{2+}. We emphasize that the elevation of cytosolic Ca^{2+} produced by these three agonists in the absence of extracellular Ca^{2+} can be as high as or higher than that which produces secretion when DMPP or K^+ are used to cause Ca^{2+}

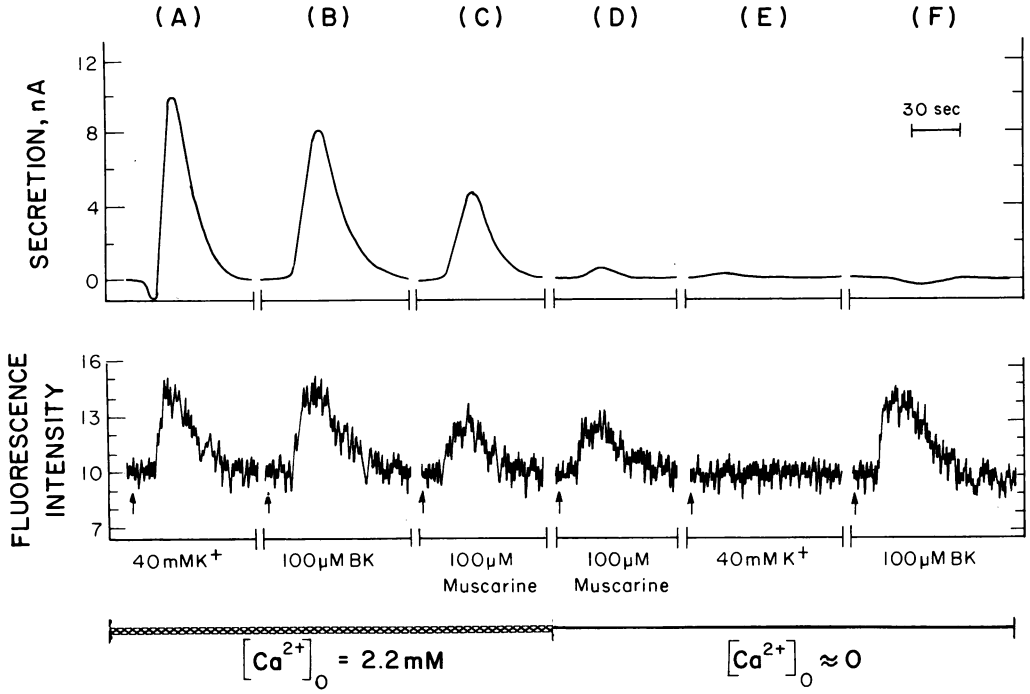

FIG. 5. Exocytosis caused by bradykinin and muscarine is dependent on external Ca^{2+}. The secretion of catecholamine was measured upon stimulation by 40 mM K^+ (traces A), 100 μM bradykinin (traces B), and 100 μM muscarine (traces C) in the presence of 2.2 mM external calcium and 100 μM muscarine (traces D), 40 mM K^+ (traces E), and 100 mM bradykinin (traces F) in the absence of extracellular calcium.

influx. UTP is unique in that it releases as much Ca^{2+} from the internal stores as do ATP, bradykinin, or muscarine but is not able to cause secretion even in the presence of external Ca^{2+}. It is difficult to quantitate, but repeated trials show that, in the presence of external Ca^{2+}, the Ca^{2+} transient caused by ATP is greater than that in the absence of external Ca^{2+}, as if in the latter case, the redistribution of Ca^{2+} is followed by influx. This same phenomenon is apparent in a publication from another laboratory (13). When the same experiments are carried out with UTP, there is no noticeable difference between the transients with or without external Ca^{2+}.

The availability of fluorescent calcium chelators that can be trapped within cells has triggered a large number of studies of changes in cytosolic calcium concentration (14). In the vast majority of these studies, there is no subcellular resolution of the calcium concentration and interpretations have to be based on the assumption that there is a single calcium concentration to which other events can be related. The central point of this report is that such an assumption is invalid. In bovine chromaffin cells, calcium released from internal stores is shown to be ineffective at triggering secretion even when the cytosolic calcium concentration measured by fura-2 is high enough to trigger substantial secretion if the calcium enters through the plasma membrane. Calcium gradients have been demonstrated in a number of cell types, and it seems most likely that the results we have observed are due to gradients that have different subcellular profiles. Apparently, entry of Ca^{2+} through the plasma membrane produces a much higher concentration of calcium at the site of exocytosis than does release of calcium from intracellular stores, which may be remote from the site of exocytosis (15). This effective compartmentalization of Ca^{2+} will very likely have important consequences for the activity of a large number of other calcium-binding proteins in the cell (16). The degree to which calcium from different sources is localized in different cell types depends on many factors including cell size. In the rat adrenal medulla, for example, it appears that muscarine can cause some release of catecholamines in the absence of external calcium (17).

There has been some controversy over the activity of muscarinic receptors in bovine adrenal medulla cells. Our experience suggests that these receptors vary in their activity from one cell batch to another (see ref. 12), but it is clear that muscarine can trigger catecholamine release from bovine chromaffin cells. It has been generally accepted that muscarinic receptors in bovine adrenal cells do not trigger secretion, although such receptors do promote phosphatidylinositol turnover (18), cGMP accumulation (19), and elevation of cytosolic Ca^{2+} (20). Kao and Schneider (20) suggested that the elevation of cytosolic Ca^{2+} by muscarine (observed with the quin-2 method) is below the threshold for secretion. These same authors had noticed (21), however, that the elevation of cytosolic Ca^{2+} by muscarine is somewhat greater in the presence of extracellular Ca^{2+} than in its absence. Our data differ in that we are able to observe secretion and show that it is entirely dependent on the influx component of the Ca^{2+} rise. The measurement of catecholamine by on-line electrochemical detection is an extremely sensitive method for measuring secretion because the secreted catecholamine is analyzed with little dilution and undetectable background secretion.

The on-line measurement of secretion from cells whose fura-2 fluorescence is being measured provides an exceptionally accurate method for comparing the cytosolic free calcium with its effect on exocytosis.

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1. Douglas, W. W. (1986) *Br. J. Pharmacol.* **34,** 451–474.
2. Knight, D. E. & Baker, P. F. (1982) *J. Membr. Biol.* **68,** 107–140.
3. Dunn, L. A. & Holz, E. W. (1983) *J. Biol. Chem.* **258,** 4989–4993.
4. Wilson, S. P. & Kirshner, N. (1983) *J. Biol. Chem.* **258,** 4994–5000.
5. Berridge, M. J. & Irvine, R. F. (1984) *Nature (London)* **312,** 315–320.
6. Chern, Y. J., Kim, K. T., Slakey, L. L. & Westhead, E. W. (1988) *J. Neurochem.* **50,** 1484–1493.
7. Grynkiewicz, G., Poenie, M. & Tsien, R. Y. (1985) *J. Biol. Chem.* **260,** 3440–3450.
8. Poenie, M., Alderton, J., Steinhardt, R. & Tsien, R. (1986) *Science* **233,** 886–889.
9. Meldolesi, J., Malgaroli, A., Wolheim, C. B. & Pozzan, T. (1987) in *In Vitro Methods for Studying Secretion*, eds. Poisner, A. & Trifaro, J. M. (Elsevier, Amsterdam), pp. 283–307.
10. Konishi, M., Olson, A., Hollingworth, S. & Baylor, S. M. (1988) *Biophys. J.* **54,** 1089–1104.
11. Meldolesi, J. & Westhead, E. W. (1989) in *Inositol Lipids in Cell Signalling*, eds. Michell, R. H., Drummond, A. H. & Downes, C. P. (Academic, London), pp 311–335.
12. Nakaki, T., Sasakawa, N., Yamamoto, S. & Kato, R. (1988) *Biochem. J.* **251,** 397–403.
13. Sasakawa, N., Nakaki, T., Yamamoto, S. & Kato, R. (1989) *J. Neurochem.* **52,** 441–447.
14. Tsien, R. Y. (1989) *Annu. Rev. Neurosci.* **12,** 227–253.
15. Volpe, P., Krause, K. H., Hashimoto, S., Zorzato, F., Pozzan, T., Meldolesi, J. & Lew, D. P. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 1091–1095.
16. Kretsinger, R. H. & Creutz, C. E. (1986) *Nature (London)* **320,** 573.
17. Harish, O. E., Kao, L. S., Raffaniello, R., Wakade, A. R. & Schneider, A. S. (1987) *J. Neurochem.* **48,** 1730–1735.
18. Fisher, S. K., Holz, R. W. & Agranoff, B. W. (1981) *J. Neurochem.* **37,** 491–497.
19. Schneider, A. S., Cline, H. T. & Lemaire, S. (1979) *Life Sci.* **24,** 1389–1394.
20. Kao, L. S. & Schneider, A. S. (1986) *J. Biol. Chem.* **261,** 4881–4888.
21. Kao, L. S. & Schneider, A. S. (1985) *J. Biol. Chem.* **260,** 2019–2022.