Pergamon

0197-0186(95)0085-2

Neurochem. Int. Vol. 28, No. 3, pp. 325–334, 1996

0197-0186/96 $15.00+0.00

CORRELATION BETWEEN SECRETAGOGUE-INDUCED Ca^{2+} INFLUX, INTRACELLULAR Ca^{2+} LEVELS AND SECRETION OF CATECHOLAMINES IN CULTURED ADRENAL CHROMAFFIN CELLS

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(Received 10 January 1995; accepted 30 May 1995)

Abstract—Catecholamine secretion induced by various secretagogues in cultured bovine chromaffin cells has been correlated with Ca^{2+} influx and intracellular Ca^{2+} concentrations. Nicotine and high K^+ caused prompt secretion of catecholamines from cells. Coincidently, both secretagogues evoked $^{45}[Ca^{2+}]$ influx with a parallel increase in free intracellular Ca^{2+} concentration, as determined by Quin 2 fluorescence. However, the rate of return of Ca^{2+} level to baseline after nicotine stimulation was more rapid than after K^+ stimulation. In comparison, stimulation with veratridine produced a slow and prolonged Ca^{2+} influx accompanied by lower levels of intracellular Ca^{2+} than those observed after nicotine or K^+ stimulation. Yet, during 15 min of stimulation, veratridine induced a substantial catecholamine release, which was larger than that obtained after nicotine or K^+ stimulations. The Ca^{2+} ionophore A23187 (1 μM) induced a pronounced increase in intracellular Ca^{2+} levels, but did not evoke any significant catecholamine release. Finally, addition of the Ca^{2+} channel blocker verapamil following stimulation, at a time when intracellular Ca^{2+} concentration was at its peak level, did not affect the rate of decline in intracellular free Ca^{2+} concentration but promptly blocked Ca^{2+} uptake and catecholamine secretion. These findings suggest that the rate of Ca^{2+} influx, rather than the absolute level of intracellular Ca^{2+} concentration, determines the rate and extent of catecholamine release.

Studies on stimulus–secretion coupling of neurotransmitters and hormones have established the pivotal role of calcium in the release process (Pollard *et al.*, 1985; Cheek and Barry, 1993). Cultured chromaffin cells secrete catecholamines in a Ca^{2+}-dependent manner by the process of exocytosis (Kilpatrick *et al.*, 1982), and can be used as a model system to study the relationships between the intracellular Ca^{2+} levels and exocytotic release. It has been suggested that in chromaffin cells the rise in cytosolic Ca^{2+} promotes the initiation of a chain of intracellular events leading to exocytosis (Burgoyne *et al.*, 1991). Various Ca^{2+}-dependent reactions have been proposed to participate in this process. Among those are phospholipase A_2 (Laychock, 1982; Moskowitz *et al.*, 1982; Petit *et al.*, 1992), Ca^{2+}/calmodulin-dependent protein phosphorylation (Treiman *et al.*, 1983), Ca^{2+}/synexin-dependent granule aggregation and fusion (Creutz *et al.*, 1992; Pollard *et al.*, 1992), calpactin-dependent fusion of granules to plasma membrane (Burgoyne *et al.*, 1991; Cheek, 1991), synapsin phosphorylation (Firestone and Browning, 1992) and Ca^{2+}-dependent dissociation of F-actin granule complexes (Trifaro *et al.*, 1992). In this regard, it has been shown that stimulation of chromaffin cells with nicotine or high K^+ increases intracellular Ca^{2+} con-

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centration from *ca* 100 to 400 nM (Baker and Knight, 1984). However, most of the Ca^{2+}-dependent processes need significantly higher Ca^{2+} concentrations for optimal activity *in vitro* (Pollard *et al.*, 1985). It is possible, however, that high concentrations of Ca^{2+} are formed at specific compartments within the cell and thereby initiate Ca^{2+}-dependent processes leading to exocytosis. Such spatial localization of intracellular Ca^{2+} has recently been demonstrated by a video-imaging technique following stimulation of chromaffin cells by agonists (Cheek *et al.*, 1993).

In order to determine the relationship between stimulus-coupled Ca^{2+} entry and the exocytotic release of catecholamines in chromaffin cells, we compared the rates of $^{45}[Ca^{2+}]$ influx with the changes in intracellular Ca^{2+} concentration as determined with the fluorescent dye Quin 2 (Tsien *et al.*, 1982). We then correlated these changes with the amount and rate of catecholamine release from the cells. The results indicate that, in chromaffin cells, Ca^{2+} influx, and not intracellular Ca^{2+} concentration, correlates best with catecholamine release, suggesting that in triggering the chain of events leading to exocytosis, Ca^{2+} ions act at or near the point of entry, as recently suggested by O'Connor *et al.* (1993).

EXPERIMENTAL PROCEDURES

Cultured chromaffin cells

Isolated chromaffin cells were obtained as described (Heldman *et al.*, 1991). Briefly, adrenal glands were perfused through the adrenal vein with 0.2% collagenase in a balanced salt solution (BSS) consisting of 125 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.2 mM $CaCl_2$, 25 mM Hepes, and 10 mM glucose, pH 7.35. The tissue was then dissected out, minced and washed on a nylon mesh (Tetco HC_3160). The tissue collected from the nylon mesh was treated with 0.2% collagenase in BSS for 30 min at 37 C. The dissociated cells were collected by centrifugation, washed four times with BSS and plated, either in 250 ml tissue culture flasks (50×10^6 cells/flask) or in 24-well plates (10^6 cells/well) in Eagle's medium containing 5% fetal calf serum (FCS), 100 μg/ml streptomycin, 5 μg ml gentamicin, 10 μg/ml cytosine arabinoside and 3 mM glutamine.

Assay of catecholamine release

Chromaffin cells, cultured for 3 days in 24-well plates, were washed with BSS and then incubated in the presence or absence of a secretagogue, as described in Results. At the appropriate times, the medium covering the cells was collected for catecholamine analysis (Kelner *et al.*, 1985). The plated cells were lysed with 2% acetic acid, frozen and thawed and the lysate centrifuged to remove cell residues. The supernatant was analyzed for total catecholamines (Kelner *et al.*, 1985). The relative amounts of catecholamine released into the medium were calculated as a percent of the total catecholamines in the particular well. No significant alteration in catecholamine release was observed in Quin 2-loaded cells compared with control cells.

Measurement of Ca^{2+} influx

Plated cells were washed with BSS, as described above, and incubated at 37 C with BSS containing $^{45}[Ca^{2+}]$ (0.5–1 μCi/well) and secretagogues where appropriate. At the end of the incubation the cells were quickly washed four times with BSS containing 2 mM $LaCl_3$ and lysed with 2% acetic acid. $LaCl_3$ completely blocks further $^{45}[Ca^{2+}]$ influx or efflux. The radioactivity accumulated in the absence of the secretagogue was subtracted from the radioactivity accumulated in the presence of secretagogue to yield the secretagogue-dependent Ca^{2+} influx (Heldman *et al.*, 1989).

Determination of intracellular Ca^{2+} concentration

Cells were collected from the tissue culture flask, washed with BSS and incubated with 20 μM Quin 2/AM (Calbiochem) in BSS. Quin 2/AM was dissolved in dimethyl sulfoxide (DMSO) and added to cells to yield a proper concentration of Quin 2/AM and a DMSO concentration not greater than 0.1%. At the end of a 1 h incubation at 37 C in the dark, the cells were collected by centrifugation, washed twice with BSS and resuspended in fresh BSS (4×10^6 cells/ml). Aliquots of 3 ml of the cell suspension were then transferred to a quartz cuvette, placed in a temperature-controlled (37 C) fluorometer and the fluorescence determined using excitation wavelength of 335 nm and emission wavelength of 495 nm. Effectors (either secretagogues or inhibitors) were added to the cuvette (in 30 μl), while measuring the fluorescence. During the entire procedure the cells were stirred by a magnetic stirrer built into the cell holder. Portions of the cell suspension were incubated in parallel without Quin 2/AM, for correction of light scattering and autofluorescence. At the end of the incubation, the cells were lysed with 3% Triton X-100 to determine the maximum fluorescence; 25 mM Mg EGTA complex, pH 8.0, was added to obtain the minimum fluorescence in the absence of free Ca^{2+}. Intracellular Ca^{2+} concentrations were calculated as previously described (Tsien *et al.*, 1982).

RESULTS

Stimulation of chromaffin cells with various secretagogues (nicotine, high K^+ and veratridine) induced a time-dependent release of catecholamines (Fig. 1). When we transformed the release at each time point to percentages, according to the maximum amount of release obtained by each secretagogue at 30 min of stimulation (considered as 100% for each secretagogue), we found that each of the secretagogues induced release by a different rate constant. Nicotine (62 μM) induced a fast and short-lasting response, reaching a plateau at 2–5 min after stimulation. The initial rate of catecholamine release following K^+ stimulation was somewhat slower, and veratridine (20 μM) produced a significantly slower initial rate of release (Fig. 1). However, when the total amount of released catecholamines during a 15-min stimulation period is considered, it is apparent that

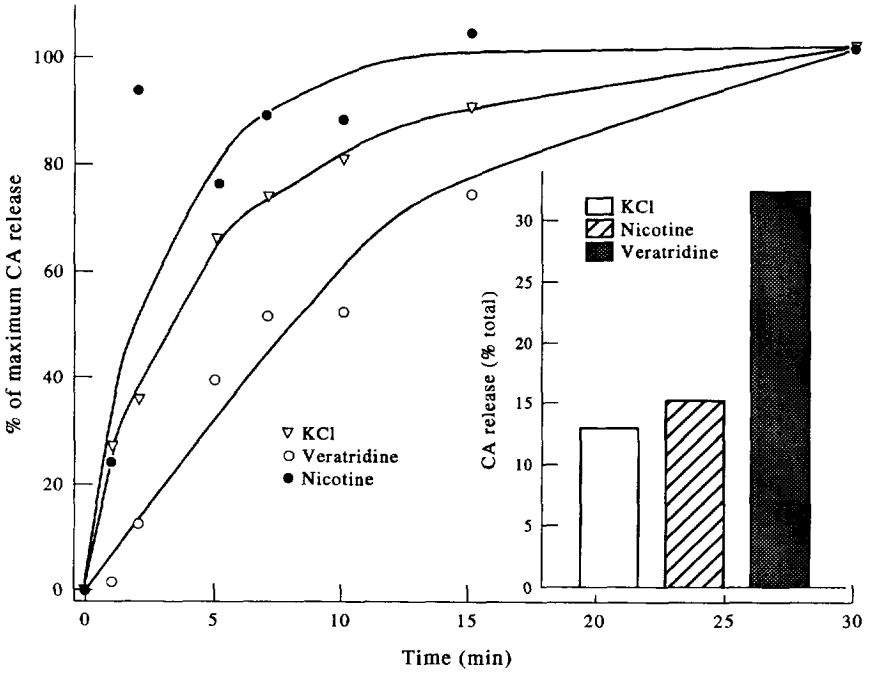

Fig. 1. Time dependency and extent of catecholamine release induced by various secretagogues from chromaffin cells in culture. Cells were preincubated in BSS at 37 C for 10 min. Nicotine (62 μM), veratridine (20 μM) or BSS, in which 50 mM KCl was substituted for 50 mM NaCl, were then added to the cells in triplicate. At various periods after the beginning of stimulation, the medium was withdrawn and analyzed for catecholamines. The amounts of catecholamines released during 30 min were considered as maximum release (100%) for each particular secretagogue. Data are from a representative experiment performed in triplicate and repeated three times. The bars in the insert represent the actual percentage of release obtained by each secretagogue after a 15-min incubation with the secretagogue.

veratridine evoked far more release than nicotine or high K^+ [Fig. 1 (inset)].

Kinetic analysis of $^{45}[Ca^{2+}]$ influx induced by nicotine, high K^+ or veratridine (Fig. 2) showed that nicotine or high K^+ activated robust- and short-lasting influxes, whereas veratridine produced a less intense but a long-lasting increase in $^{45}[Ca^{2+}]$ influx. The data in Fig. 2 represent secretagogue-dependent $^{45}[Ca^{2+}]$ uptake, calculated by subtracting basal uptake from uptake in the presence of secretagogues. In the case of nicotine, $^{45}[Ca^{2+}]$ uptake ceased after 2 min, probably due to receptor desensitization and $^{45}[Ca^{2+}]$ efflux, which follows the rise in intracellular Ca^{2+}. On the other hand, basal $^{45}[Ca^{2+}]$ uptake continued to rise. Therefore, after 2 min, nicotine-dependent $^{45}[Ca^{2+}]$ uptake started to decline. By contrast, in the case of veratridine, stimulated Ca^{2+} uptake continued to rise more rapidly than the basal for a longer period of time, leading to a prolonged rise in veratridine-dependent $^{45}[Ca^{2+}]$ uptake.

Utilizing Quin 2/AM, we studied how the intracellular Ca^{2+} concentration is related to the Ca^{2+} influx on one hand, and to the rate of catecholamine release on the other hand. As shown in Fig. 3(A), addition of nicotine to the cells induced a quick increase in intracellular Ca^{2+} concentration, reaching its peak level in 10–20 s. A gradual decline of Ca^{2+} concentration to its resting level persisted 3–6 min after stimulation with the agonist [Table 1 and Fig. 3(A)]. In some cases, the new baseline was somewhat higher than that observed before stimulation. By comparison, K^+ stimulation [Fig. 3(B)] led to a rise in intracellular Ca^{2+} followed by a decay that lasted significantly longer (12–16 min) than that observed in nicotine-stimulated cells. In many experiments, the new baseline was higher than that observed before stimulation.

As shown in Fig. 4, stimulation with veratridine also caused a rise in Ca^{2+} concentration. However, this increase was much slower (75–90 s) than the rise observed after stimulation with either high K^+ or nicotine (cf. Fig. 3), and its magnitude was generally lower. The peak values for Ca^{2+} concentration after various stimulations, as well as their rise and decay

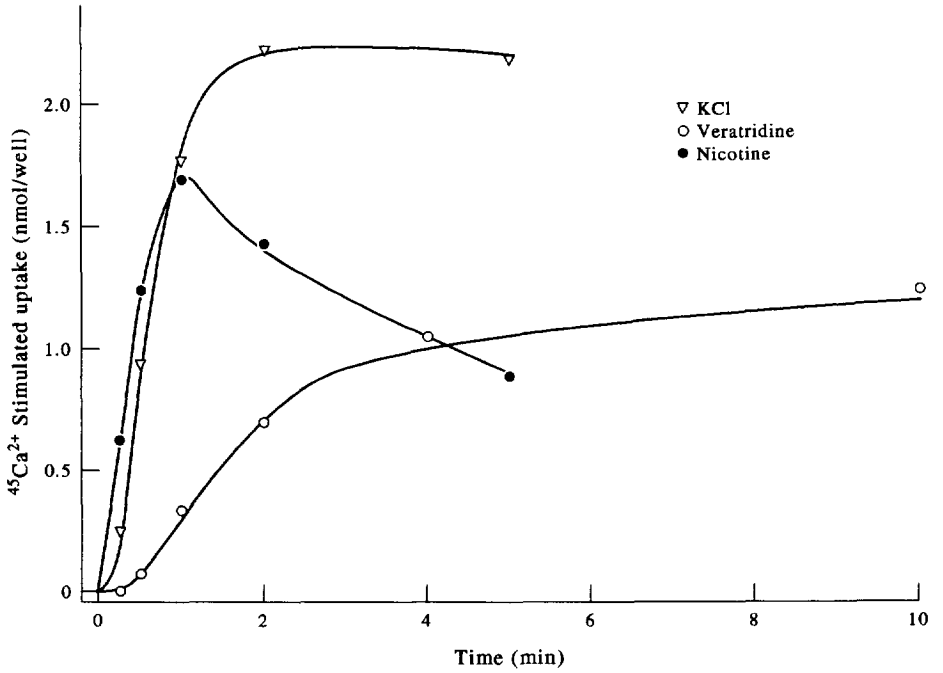

Fig. 2. Kinetics of the secretagogue-dependent $^{45}[Ca^{2+}]$ uptake to chromaffin cells in culture. Cells were preincubated at 37°C in BSS 10 min prior to the addition of $^{45}[Ca^{2+}]$ together with nicotine (62 μM), veratridine (20 μM) or KCl (50 mM). At various times after the simultaneous addition of $^{45}[Ca^{2+}]$ and secretagogue, cells were washed and radioactivity in cell lysate counted. Each point represents the mean difference between the uptake observed in the presence of secretagogue and that observed in its absence. Data are from a representative experiment replicated three times.

times, are summarized in Table 1, and the real-time kinetics is displayed in Fig. 5. Veratridine induced a slow increase in Ca^{2+} concentration that was lower in magnitude than that produced by nicotine or high K^+. However, 3–10 min after stimulation, Ca^{2+} concentrations reached similar levels to those induced by high K^+. However, as shown in Fig. 1 (inset), the release induced by high K^+ is smaller than the release induced by veratridine over the same time period. Thus, intracellular Ca^{2+} concentrations do not seem to correlate quantitatively with the magnitude of catecholamine secretion.

These findings raise the possibility that the site for regulation of secretion by Ca^{2+} is at the site of entry, i.e. at the Ca^{2+}-channel level. We therefore investigated the effect of verapamil, an agent known to cause an abrupt blockade of Ca^{2+} influx by inhibiting the Ca^{2+} channel. We confirmed previous studies (Corcoran and Kirshner, 1983; Knight and Kesteven, 1983) that verapamil (5×10^{-4} M) inhibits $^{45}[Ca^{2+}]$ influx stimulated by high K^+ or nicotine. Using the same paradigm, we found that addition of verapamil, a short time after stimulation with high K^+ or nicotine, completely inhibits catecholamine secretion. For example, in the case of stimulation by nicotine, 6% of the catecholamines were released in the first

Table 1. Parameters related to changes in Ca^{2+} concentration following various stimulations

Stimulus	Peak value Ca^{2+} (nM mean ± SE) (*n*)	Time to peak (range in s)	Time to baseline (range in min)
Resting cells	142 ± 6 (43)		
Nicotine	318 ± 20 (13)	10–20	3–6
KCl (50 mM)	329 ± 18 (13)	15–25	12–16
Veratridine	256 ± 46 (12)	90–150	6–10
A23187	249 ± 35 (4)	90–210	

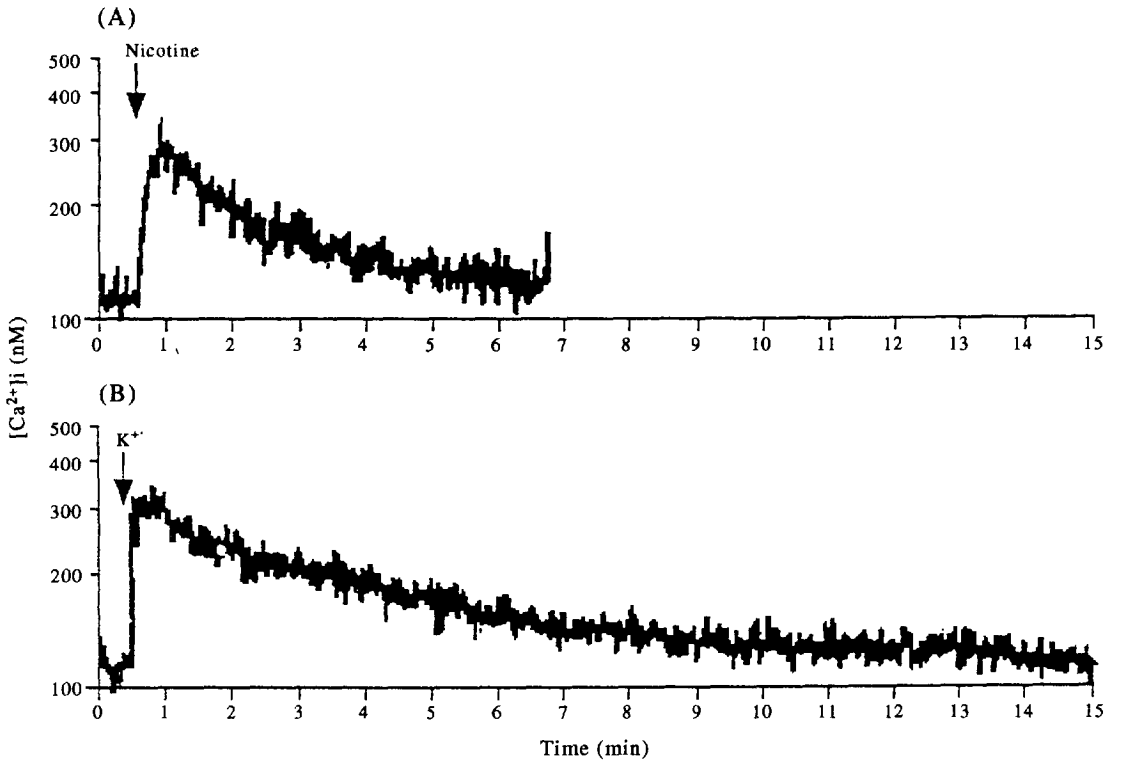

Fig. 3. Intracellular Ca^{2+} levels in chromaffin cells at various time intervals after stimulation with (A) nicotine or (B) high K^+. Quin-loaded cells were stimulated with nicotine (62 μM) or KCl (25 mM). Fluorescence was continuously determined during and after stimulation. Intracellular Ca^{2+} concentrations were calculated from the fluorescence values by considering the maximum and minimum fluorescence values obtained at the end of each experiment (Tsien *et al.*, 1982) (see Table 1 for mean values and statistical analysis).

minute of incubation. An additional 8% were released in the second minute after stimulation. Addition of verapamil 1 min after stimulation with nicotine, completely abolished the secretion of catecholamines during the second minute after stimulation. This observation allows examination of whether secretion is coupled to the elevated intracellular Ca^{2+} concentration or to Ca^{2+} influx. If intracellular Ca^{2+} level is directly related to Ca^{2+} influx, then verapamil should induce an abrupt reduction in Ca^{2+} concentration coincident with its blockade of secretion. However, as shown in Fig. 6, when verapamil (500 μM) was added after Ca^{2+} levels have reached the peak value, no immediate drop in intracellular Ca^{2+} concentration could be observed. Similar results were obtained by addition of 2 mM La^{3+}, which is known as a specific Ca^{2+}-channel blocker. Thus, the important site for Ca^{2+} regulation of secretion would appear to be at the level of the channel rather than at the level of intracellular Ca^{2+} concentration *per se*.

To test this conclusion, we utilized the Ca^{2+} ionophore A23187 to introduce Ca^{2+} ions by a route exclusive of the Ca^{2+} channel. When the Ca^{2+} ionophore A23187 (1 μM) was added to resting chromaffin cells, a significant increase in Ca^{2+} concentration was seen within 1.5–3.5 min (Table 1). However, this increase was not accompanied by a significant catecholamine release (data not shown; see also Morita *et al.*, 1985), suggesting again that the rise in cytosolic Ca^{2+} alone is not sufficient to trigger the exocytotic process.

DISCUSSION

The conventional concept of how calcium controls secretion in chromaffin cells is based on several obser-

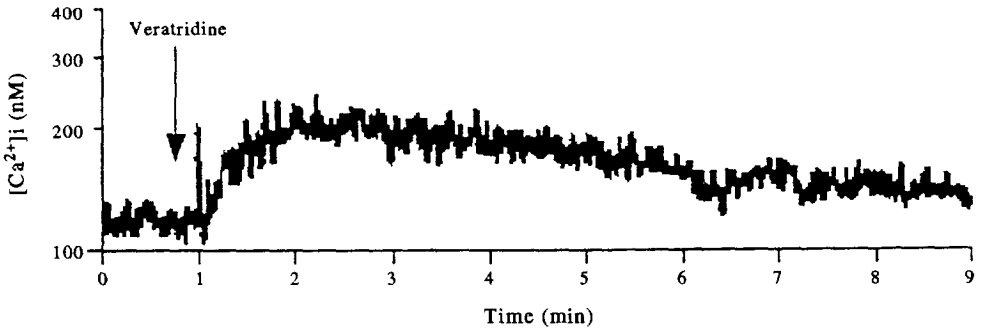

Fig. 4. Intracellular Ca^{2+} levels in chromaffin cells at various time intervals after veratridine stimulation. Veratridine was added to the cell suspension to yield a final concentration of 20 μM (see Table 1 for statistical analysis).

vations: (i) extracellular Ca^{2+} is essential to trigger the secretory process; (ii) Ca^{2+} influx is activated upon cell stimulation; (iii) Ca^{2+} influx is followed by catecholamine release (Holz *et al.*, 1982; Kilpatrick *et al.*, 1982; Corcoran and Kirshner, 1983). It has therefore been assumed that Ca^{2+} that enters the cell during stimulation causes cytosolic Ca^{2+} to rise to such a concentration that it initiates Ca^{2+}-dependent processes that are part of the release mechanism. The experimental results presented here demonstrate that even when cytosolic Ca^{2+} is elevated, release does not necessarily occur. This is in agreement with our previous results demonstrating an insignificant release of catecholamines from chromaffin cells treated with the Ca^{2+} ionophore A23187 (Morita *et al.*, 1985). Yet another Ca^{2+} ionophore, ionomycin, induces Ca^{2+} influx and some catecholamine secretion (Kilpatrick *et al.*, 1980). However, the release induced by ionomycin is much smaller than that induced by nicotine or acetylcholine and may be related to the effect of this Ca^{2+} ionophore on K^+ currents (Estación, 1991). To explain our observations we propose that the

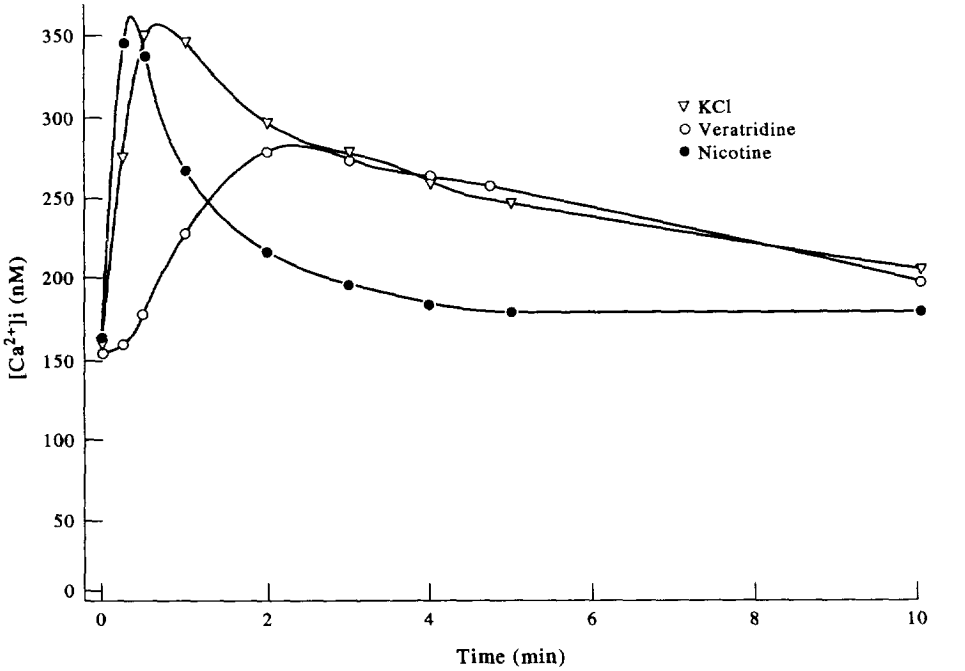

Fig. 5. Intracellular Ca^{2+} concentrations in chromaffin cells at various times after stimulation with secretagogues. Each point represents the mean values for the particular time after stimulation, calculated from at least five different experiments. The concentrations of the secretagogues were: nicotine (62 μM), veratridine (20 μM) and KCl (25 mM).

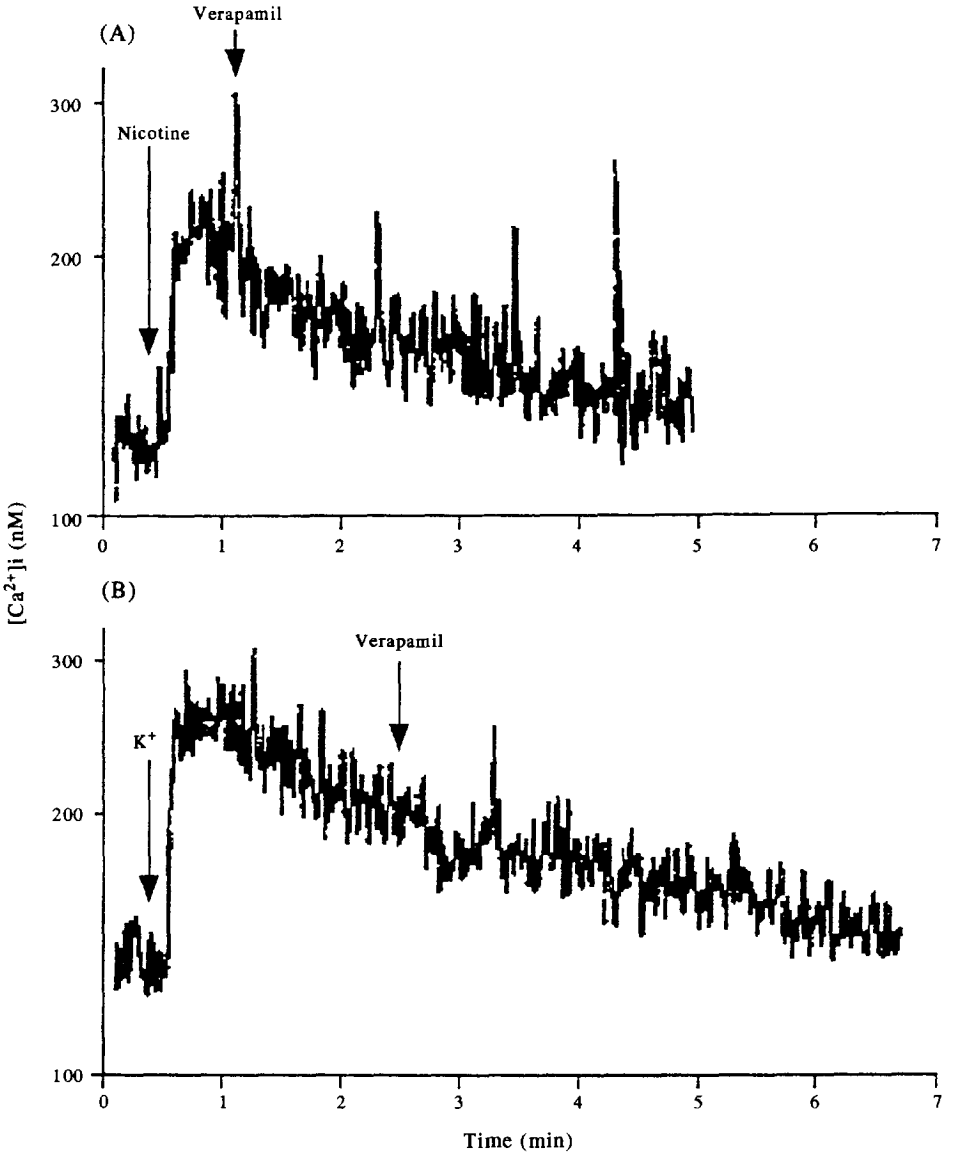

Fig. 6. Effect of verapamil on intracellular Ca^{2+} concentrations. Verapamil (500 μM) was added to cells stimulated with (A) nicotine (62 μM) and (B) KCl (25 mM). The figure shows representative traces.

release of catecholamines is initiated by Ca^{2+}, entering the cell from the extracellular medium, acting at/or near the site of Ca^{2+} entry.

This hypothesis can be examined by comparing the rate of catecholamine release (regardless of the choice of secretagogue) with either the changes of internal free Ca^{2+} concentration or with the rates of $^{45}[Ca^{2+}]$ influx. As shown in Fig. 7(A), there is a general tendency for more release to occur at higher concentrations of intracellular free Ca^{2+}. However, the two parameters are poorly correlated ($r = 0.3088$). For analysis of the correlation, data for high K^+, veratridine and nicotine were simply pooled. There is some clustering of points for a particular secretagogue, and this may be of importance in terms of secretagogue-specific mechanism. However, this clustering of points for each secretagogue and the lack of correlation between intracellular Ca^{2+} concentration and catecholamine release suggest that elevation of intracellular Ca^{2+} concentration alone is not sufficient

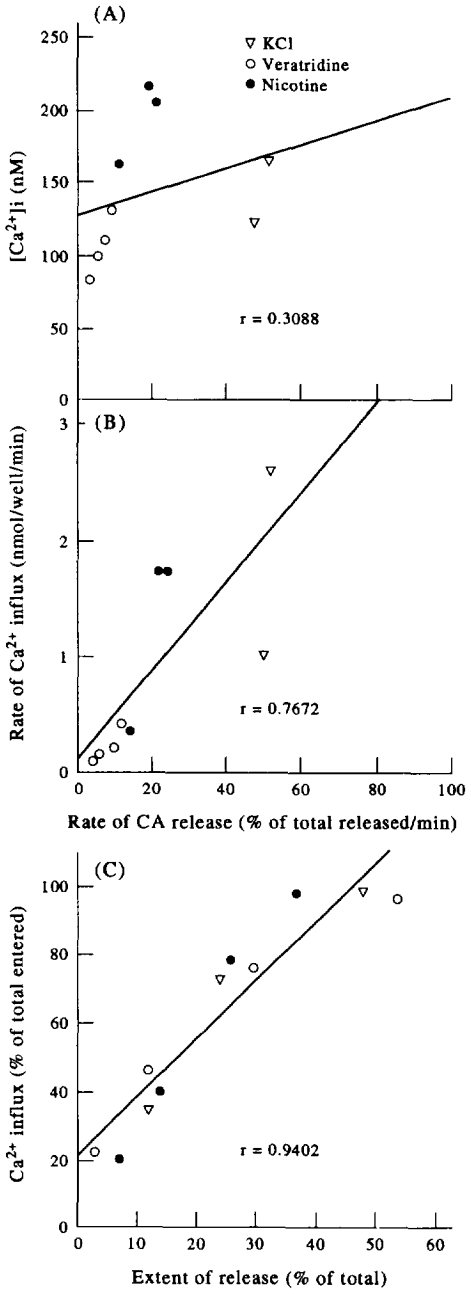

Fig. 7. Relationship between catecholamine release, intracellular Ca^{2+} levels and rate of Ca^{2+} influx. (A) Correlation between intracellular free Ca^{2+} concentration and the rate of catecholamine release induced by veratridine, KCl or nicotine. (B) Correlation between the rate of Ca^{2+} influx (using the $^{45}[Ca^{2+}]$ method) and the rate of catecholamine release induced by the same secretagogues. (C) Correlation between the Ca^{2+} influx and the extent of catecholamine release under the same conditions. The regression analyses as shown by the r values are significant, $P < 0.05$. Small r values are indicative of a poor correlation between the parameters.

to trigger the exocytotic mechanism. In contrast, there seems to be a better correlation between the rates of $^{45}[Ca^{2+}]$ influx and the magnitude of catecholamine release ($r = 0.7672$) [see Fig. 7(B)]. As shown in Fig. 7(C), a comparison of the relative extent of release with the proportion of total Ca^{2+} influx at any given time reveals a close correlation ($r = 0.9402$). Most of the Ca^{2+} influx occurs within the first few minutes after stimulation (Fig. 2), and it is after this period that the catecholamine rate of release begins to decline. Thus, it seems that release of catecholamines is dependent on the rate of Ca^{2+} influx and not on the level of intracellular Ca^{2+} *per se*.

It is worthwhile to note that this analysis explains the hitherto enigmatic aspects of veratridine-stimulated secretion and the accompanying changes in intracellular Ca^{2+} concentration. Veratridine causes a slow but continuous Ca^{2+} influx. According to our hypothesis, the cell should thus secrete slowly and continuously, as it indeed does. The intracellular Ca^{2+} concentration after stimulation with veratridine remains relatively low (possibly due to sequestration), but without a decreasing effect on release. This observation is consistent with the concept that Ca^{2+} concentration is not directly related to rate of secretion.

We would like to propose that a site at/or near the Ca^{2+} channel must be occupied by Ca^{2+} in order for release to occur (see also O'Connor *et al.*, 1993). Our observations with verapamil, mentioned above, are also consistent with this concept. Furthermore, this model also explains why high K^+ induces catecholamine release that ceases even at a time when cytosolic Ca^{2+} levels are still high. It is known that Ca^{2+} channels are inactivated within a short time after K^+ stimulation causing Ca^{2+} influx to cease (Heldman *et al.*, 1991). When Ca^{2+} influx ceases, the high local Ca^{2+} concentration in the immediate vicinity of the channel is decreased, leading to a reduction in the rate of catecholamine release. Indeed, imaging of intracellular free Ca^{2-} demonstrates that after stimulation, the rise of intracellular Ca^{2+} concentration begins at the periphery of the cell (Neher and Augustine, 1992). Moreover, it has been suggested that following depolarization (Augustine and Neher, 1992), as well as following agonist stimulation (Cheek *et al.*, 1993), spatial gradients of intracellular Ca^{2+} concentration are formed. Our data suggest that the spatial gradients must begin at the point of Ca^{2+} entry and this could very well be in proximity to the site where Ca^{2-} ions initiate the processes leading to exocytotic release. A hypothetical Ca^{2+}-binding protein is believed to be present at the site of Ca^{2+} action in exocytosis (Howell *et al.*, 1987; Gomperts *et al.*,

1988; Monck and Fernández, 1992; Okano *et al.*, 1993), and it is thought to be close to the fusion site (Bennett *et al.*, 1992; Monck and Fernández, 1992; White, 1992). Based on caged calcium studies, it has been estimated that the affinity of this hypothetical protein for calcium is in the low 50–200 μM range (Augustine and Neher, 1992; Bittner and Holz, 1992; Neher and Zucker, 1993; von Rüden and Neher, 1993). Our study shows that the average intracellular Ca^{2+} concentration obtained following stimulation with K^+, nicotine or veratridine is well below this value. The high Ca^{2+} concentrations (50–200 μM) needed to activate such a hypothetical Ca^{2+}-binding protein can only be obtained locally at or near the Ca^{2+} points of entry into the cell. In conclusion, the present findings suggest that the regulation of catecholamine secretion by Ca^{2+} occurs at or close to the sites of Ca^{2+} entry.

Acknowledgement—This work was partially supported by grant no. DA-6265 from the National Institute of Drug Abuse, U.S.A., given to Zvi Vogel.

REFERENCES

Augustine G. J. and Neher E. (1992) Calcium requirements for secretion in bovine chromaffin cells. *J. Physiol.* (Lond.) **450,** 247–271.

Baker P. F. and Knight D. E. (1984) Calcium control of exocytosis in bovine adrenal medullary cells. *TINS* **7,** 120–126.

Bennett M. K., Calakos N. and Scheller R. H. (1992) Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. *Science* **257,** 255–259.

Bittner M. A. and Holz R. W. (1992) Kinetic analysis of secretion from permeabilized adrenal chromaffin cells reveals distinct components. *J. Biol. Chem.* **267,** 16,219–16,225.

Burgoyne R. D., Handel S. E., Morgan A., Rennison M. E., Turner M. D. and Wilde C. J. (1991) Calcium, the cytoskeleton and calpactin (anexin II) in exocytotic secretion from adrenal chromaffin and mammary epithelial cells. *Biochem. Soc. Trans.* **19,** 1085–1090.

Cheek T. R. (1991) Calcium signaling and the triggering of secretion in adrenal chromaffin cells. *Pharmac. Ther.* **52,** 173–189.

Cheek T. R. and Barry V. A. (1993) Stimulus-secretion coupling in excitable cells: a central role for calcium. *J. Exp. Biol.* **184,** 183–196.

Cheek T. R., Morgan A., O'Sullivan J., Moreton R. B., Berridge M. J. and Burgoyne R. D. (1993) Spatial localization of agonist-induced entry in bovine adrenal chromaffin cells. *J. Cell Sci.* **105,** 913–921.

Corcoran J. J. and Kirshner N. (1983) Inhibition of calcium uptake, sodium uptake, and catecholamine secretion by methoxyverapamil (D600) in primary cultures of adrenal medulla cells. *J. Neurochem.* **40,** 1106–1109.

Creutz C. E., Kambouris N. G., Snyder S. L., Hamman H. C., Nelson M. R., Liu W. and Rock P. (1992) Effects of the expression of mammalian anexins in yeast secretory mutants. *J. Cell Sci.* **103,** 1177–1192.

Estación M. (1991) Acute electrophysiological responses of bradykinin-stimulated human fibroblasts. *J. Physiol.* (Lond.) **436,** 603–620.

Firestone J. A. and Browning M. D. (1992) Synapsin II phosphorylation and catecholamine release in bovine adrenal chromaffin cells: additive effects of histamine and nicotine. *J. Neurochem.* **58,** 441–447.

Gomperts B. D., Cockroft S., Howell T. W., Nusse O. and Tatham P. E. (1988) The dual effector system for exocytosis in mast cells: obligatory requirement for both Ca^{2+} and GTP. *Biosci. Rep.* **7,** 369–381.

Heldman E., Levine M., Raveh L. and Pollard H. B. (1989) Barium ions enter chromaffin cells via voltage-dependent calcium channels and induce secretion by a mechanism independent of calcium. *J. Biol. Chem.* **264,** 7914–7920.

Heldman E., Levine M., Morita K. and Pollard H. B. (1991) Osmotic strength differentiates between two types of calcium transport pathways regulating catecholamine secretion from cultured bovine chromaffin cells. *Biochim. Biophys. Acta* **1091,** 417–425.

Holz R. W., Senter R. A. and Frye R. A. (1982) Relationship between Ca^{2+} uptake and catecholamine secretion in primary dissociated cultures of adrenal medulla. *J. Neurochem.* **39,** 635–646.

Howell T. W., Cockroft S. and Gomperts B. D. (1987) Essential synergy between Ca^{2+} and guanine nucleotides in exocytotic secretion from permeabilized rat mast cells. *J. Cell Biol.* **105,** 191–197.

Kelner K. L., Levine R. A., Morita K. and Pollard H. B. (1985) A comparison of trihydroxyindole and HPLC/electrochemical methods for catecholamine measurement in adrenal chromaffin cells. *Neurochem. Int.* **7,** 373–378.

Kilpatrick D. L., Ledbetter F. W., Carson K. A., Kirshner A. G., Slepetis R. and Kirshner N. (1980) Stability of bovine adrenal medulla cells in culture. *J. Neurochem.* **35,** 679–692.

Kilpatrick D. L., Slepetis R. J., Corcoran J. J. and Kirshner N. (1982) Calcium uptake and catecholamine secretion by cultured bovine adrenal medulla cells. *J. Neurochem.* **38,** 427–435.

Knight D. E. and Kesteven N. T. (1983) Evoked transient intracellular free Ca^{2+} changes and secretion in isolated bovine adrenal medullary cells. *Proc. R. Soc. Lond.* **218,** 177–199.

Laychock S. G. (1982) Phospholipase A_2 activity in pancreatic islets is calcium-dependent and stimulated by glucose. *Cell Calcium* **3,** 43–54.

Monck J. R. and Fernández J. M. (1992) The exocytotic fusion pore. *J. Cell Biol.* **119,** 1395–1404.

Morita K., Brocklehurst K. W., Tomares S. M. and Pollard H. B. (1985) The phorbol ester TPA enhances A23187– but not carbachol—and high K^+-induced catecholamine secretion from cultured bovine adrenal chromaffin cells. *Biochem. Biophys. Res. Chem.* **129,** 511–516.

Moskowitz N., Schook W. and Puszkin S. (1982) Interaction of brain synaptic vesicles induced by endogenous Ca^{2+}-dependent phospholipase A_2. *Science* **216,** 305–307.

Neher E. and Augustine G. J. (1992) Calcium gradients and buffers in bovine chromaffin cells. *J. Physiol.* (Lond.) **450,** 273–301.

Neher E. and Zucker R. S. (1993) Multiple calcium-dependent processes related to secretion in bovine chromaffin cells. *Neuron* **10,** 21–30.

O'Connor V. M., Shamotienko O., Grishin E. and Betz H. (1993) On the structure of the "synaptosecretosome": evidence for a neurexin/synaptotagmin/syntaxin/Ca^{2+} channel complex. *FEBS Lett.* **326,** 255–260.

Okano K., Monck J. R. and Fernández J. M. (1993) GTP-γ-S stimulates exocytosis in patch-clamped rat melanotrophs. *Neuron* **11,** 165–172.

Petit K., Miserez B., De Block J., Van Dessel G. and De Potter W. (1992) The presence of phospholipase A_2 in bovine adrenal medulla: arguments for more than one type of phospholipase A_2. *Biochim. Biophys. Acta* **1125,** 150–156.

Pollard H. B., Rojas E. and Burns A. L. (1992) Synexin (anexin VII) and membrane fusion during the process of exocytotic secretion. *Progr. Brain Res.* **92,** 247–255.

Pollard H. B., Ornberg R., Levine M., Kelner K., Morita K., Levine R., Forsberg E., Brocklehurst K. W., Duong L., Lelkes P., Heldman E. and Youdim M. B. H. (1985) Hormone secretion by exocytosis with emphasis on information from the chromaffin cell system. *Vitamins Hormones* **42,** 109–196.

von Rüden L. and Neher E. (1993) A Ca-dependent early step in the release of catecholamines from adrenal chromaffin cells. *Science* **262,** 1061–1065.

Treiman M., Weber W. and Gratzl M. (1983) 3′,5′-Cyclic adenosine monophosphate- and Ca^{2+}/calmodulin-dependent endogenous protein phosphorylation activity in membranes of the bovine chromaffin secretory vesicles: identification of two phosphorylated components as tyrosine hydroxylase and protein kinase regulatory subunit type II. *J. Neurochem.* **40,** 661–669.

Trifaro J. M., Rodríguez del Castillo A. and Vitale M. L. (1992) Dynamic changes in chromaffin cell cytoskeleton as prelude to exocytosis. *Molec. Neurobiol.* **6,** 339–358.

Tsien R. Y., Pozzan T. and Rink T. J. (1982) Calcium homeostasis in intact lymphocytes: cytoplasmic free calcium monitored with a new, intracellularly trapped fluorescent indicator. *J. Cell Biol.* **94,** 325–334.

White J. (1992) Membrane fusion. *Science* **258,** 917–924.