Journal of Neurochemistry
Raven Press, Ltd., New York

GTP and Ca^{2+} Modulate the Inositol 1,4,5-Trisphosphate-Dependent Ca^{2+} Release in Streptolysin O-Permeabilized Bovine Adrenal Chromaffin Cells

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Abstract: The inositol 1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release was studied using streptolysin O-permeabilized bovine adrenal chromaffin cells. The IP_3-induced Ca^{2+} release was followed by Ca^{2+} reuptake into intracellular compartments. The IP_3-induced Ca^{2+} release diminished after sequential applications of the same amount of IP_3. Addition of 20 μM GTP fully restored the sensitivity to IP_3. Guanosine 5'-*O*-(3-thio)triphosphate (GTPγS) could not replace GTP but prevented the action of GTP. The effects of GTP and GTPγS were reversible. Neither GTP nor GTPγS induced release of Ca^{2+} in the absence of IP_3. The amount of Ca^{2+} whose release was induced by IP_3 depended on the free Ca^{2+} concentration of the medium. At 0.3 μM free Ca^{2+}, a half-maximal Ca^{2+} release was elicited with ~0.1 μM IP_3. At 1 μM free Ca^{2+}, no Ca^{2+} release was observed with 0.1 μM IP_3; at this Ca^{2+} concentration, higher concentrations of IP_3 (0.25 μM) were required to evoke Ca^{2+} release. At 8 μM free Ca^{2+}, even 0.25 μM IP_3 failed to induce release of Ca^{2+} from the store. The IP_3-induced Ca^{2+} release at constant low (0.2 μM) free Ca^{2+} concentrations correlated directly with the amount of stored Ca^{2+}. Depending on the filling state of the intracellular compartment, 1 mol of IP_3 induced release of between 5 and 30 mol of Ca^{2+}. **Key Words:** Inositol 1,4,5-trisphosphate—Ca^{2+} release—Streptolysin O—Bovine adrenal chromaffin cells—GTP—Guanosine 5'-*O*-(3-thio)triphosphate. **Föhr K. J. et al.** GTP and Ca^{2+} modulate the inositol 1,4,5-trisphosphate-dependent Ca^{2+} release in streptolysin O-permeabilized bovine adrenal chromaffin cells. *J. Neurochem.* **56,** 665–670 (1991).

Intact bovine adrenal chromaffin cells, depending on the stimulus, use extracellular and/or intracellular sources to increase the cytoplasmic free Ca^{2+} concentration (cf. O'Sullivan and Burgoyne, 1989). Release of intracellular stored Ca^{2+} can be induced from the endoplasmic reticulum by inositol 1,4,5-trisphosphate (IP_3), which is produced by the activation of phospholipase C at the plasma membrane (Berridge and Irvine, 1984, 1989). In digitonin-permeabilized adrenal chromaffin cells, high concentrations of IP_3 were required to induce release of Ca^{2+}. Furthermore, a second dose of IP_3 failed to induce release of Ca^{2+}, and the Ca^{2+} released could not be taken up again by the compartment (Stoehr et al., 1986; Kao, 1988). These findings argue against a major contribution of the IP_3-sensitive Ca^{2+} compartment during stimulation of adrenal chromaffin cells. One possibility is that digitonin used in the previous studies for permeabilization may cause disintegration of intracellular membranes and thus explain unsatisfactory IP_3-induced Ca^{2+} release. In the present study we therefore permeabilized the cells with streptolysin O (SLO) from β-hemolytic streptococci whose action is restricted to the plasma membrane (Hugo et al., 1986; Bhakdi and Tranum-Jensen, 1987; Ahnert-Hilger et al., 1989*a*). This pore-forming protein has been introduced as an excellent tool to investigate the role of Ca^{2+} during exocytosis (cf. Ahnert-Hilger et al., 1989*b*).

Using SLO-permeabilized cells and a Ca^{2+}-specific electrode, we found that release of Ca^{2+} is induced by low concentrations of IP_3 and that the amount of released Ca^{2+} is comparable to that observed in other secretory cells (cf. Berridge, 1987). Furthermore, we demonstrate here that the IP_3-induced Ca^{2+} release is

Received February 23, 1990; final revised manuscript received July 3, 1990; accepted August 14, 1990.

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Abbreviations used: GTPγS, guanosine 5'-*O*-(3-thio)triphosphate; IP_3, inositol 1,4,5-trisphosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SLO, streptolysin O.

modulated by GTP and depends on the amount of stored Ca^{2+} as well as on the cytoplasmic free Ca^{2+} concentration.

MATERIALS AND METHODS

The pore-forming protein SLO was purified as previously described (Bhakdi et al., 1984) and kindly provided by S. Bhakdi (Gießen, F.R.G.). IP_3 was from Boehringer (Mannheim, F.R.G.). GTP and guanosine 5'-*O*-(3-thio)triphosphate (GTPγS) were from Sigma (München, F.R.G.). Ca^{2+}-selective membranes containing the neutral carrier ETH 129 were kindly provided by W. Simon (ETH, Zürich, Switzerland). Azur A was from Fluka (Neu-Ulm, F.R.G.). All other reagents were of analytical grade.

Cell culture, permeabilization, and Ca^{2+} content measurements

Bovine adrenal glands were obtained from the local slaughterhouse. Chromaffin cells were prepared and put into short-time cultures as described previously (Livett, 1984) with some modifications (Stecher et al., 1989). Isolated chromaffin cells (1×10^7) were plated on 60-mm-diameter dishes and used after 2 days of cultivation. The cells were washed three times with medium A of the following composition (in mmol/L): 150 KCl, 5 NaN_3, 20 3-(*N*-morpholino)propanesulfonic acid (MOPS; pH 7.2), and 1 EGTA. For permeabilization, the cells were suspended in the same medium containing 300 hemolytic units of SLO/10^7 cells on ice and then incubated for 10 min at 30°C. Permeabilization was controlled by counting the azur A-stained cells (Föhr et al., 1989). After permeabilization the cells were washed three times by centrifugation (2 min, 400 g_{av}) in medium A without EGTA. The Ca^{2+} content was measured with a Ca^{2+}-specific electrode in 0.4–0.5 ml of medium B (medium A without EGTA but with 2 m*M* Mg^{2+}-ATP) as described previously (Föhr et al., 1989).

RESULTS AND DISCUSSION

In the presence of 2 m*M* Mg^{2+}-ATP and 5 m*M* NaN_3, which blocks Ca^{2+} uptake by mitochondria (Fulceri et al., 1989), Ca^{2+} was rapidly taken up by the permeabilized adrenal chromaffin cells. A low concentration of IP_3 (here, 0.15 μ*M*) was sufficient to induce Ca^{2+} release at Ca^{2+} concentrations occurring in resting cells (Fig. 1A). The Ca^{2+} release was followed by an immediate reuptake, and subsequent applications of the same amount of IP_3 (0.15 μ*M*) resulted in a stepwise decrease in the amount of released Ca^{2+} (Fig. 1A). This decrease in the amount of released Ca^{2+} was also observed if the cells had been left for a longer time before addition of IP_3 (Fig. 1B), a result indicating that the apparent desensitization does not reflect a gradually falling Ca^{2+} content baseline. As determined in a series of experiments, a second addition of IP_3 elicited ~50% of the signal obtained by the first application (Fig. 1C). A similar desensitization occurring during repeated application of nonsaturating IP_3 concentrations was also reported by Stoehr et al. (1986) for chromaffin cells and by Rossier et al. (1987) for adrenocortical cells. This apparent desensitization was completely

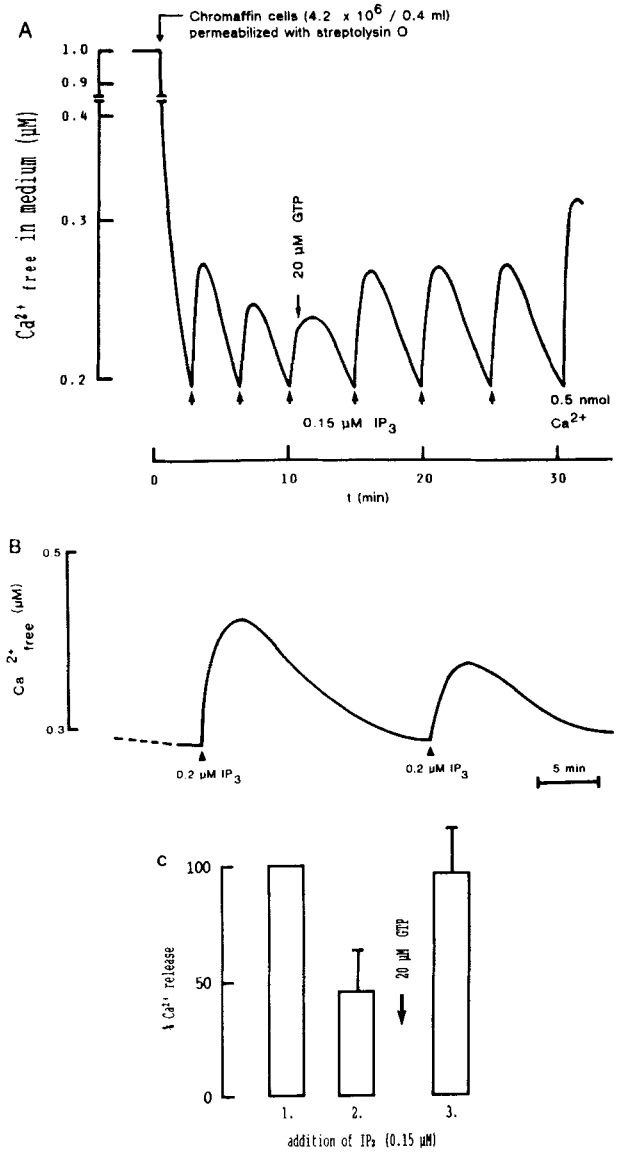

FIG. 1. Effect of GTP on IP_3-induced Ca^{2+} release. Ca^{2+} release induced by IP_3 from permeabilized cells was studied without additional loading of the Ca^{2+} store (as in Fig. 4). Sequential additions of 0.15 μ*M* IP_3 led to a decreased Ca^{2+} release. **A:** After a single dose of 20 μ*M* GTP, the amounts of Ca^{2+}, whose release was induced by IP_3, were similar in size, as observed during the first release. **B:** The IP_3-induced Ca^{2+} release also decreases when the IP_3-sensitive Ca^{2+} compartment is maximally filled by additional Ca^{2+} (see Fig. 4A) and left longer to obtain a flat baseline. **C:** Summary of 17 independent experiments carried out as in A with the exception that addition of GTP was started after the second IP_3 application. Under these conditions (without maximal filling of the Ca^{2+} compartment), 100% corresponds to the release of 1.0 ± 0.2 nmol of Ca^{2+}/10^7 cells (mean ± SD). Data are mean ± SD (bars) values.

overcome by a single addition of 20 μ*M* GTP (Figs. 1A and C, 2B, and 3). Therefore, if not stated otherwise, 20 μ*M* GTP was included from the beginning of the experiments to avoid desensitization on repeated IP_3 applications. Whereas addition of 20 μ*M* GTP im-

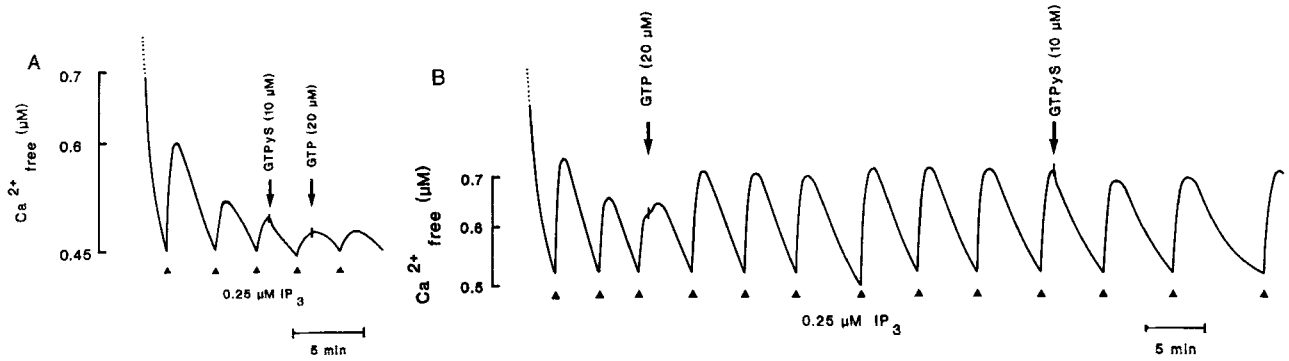

FIG. 2. GTPγS blocks the GTP effect on IP_3-induced Ca^{2+} release. GTPγS (10 μM) completely blocks the effect of GTP (20 μM) on the IP_3-induced Ca^{2+} release when added before GTP (**A**) but has no effect on the IP_3-induced Ca^{2+} release when added after addition of GTP (**B**). These results are representative of three similar experiments.

mediately restored sensitivity to IP_3, 10 μM GTP caused a stepwise recovery of the maximal IP_3 response within 30 min (data not shown).

Neither GTP itself nor the stable analogue GTPγS induced release of Ca^{2+} on its own. However, the effect of GTP on the desensitization was completely blocked when GTPγS (10 μM) was added before GTP (20 μM) (Fig. 2A). On the other hand, when desensitization was first abolished by GTP, addition of GTPγS could not restore desensitization (Fig. 2B). The effect of GTP was reversible (Fig. 3). When permeabilized cell preparations were first analyzed as shown in Fig. 1A and C and subsequently washed twice in 150 mM KCl, 20 mM MOPS (pH 7.2), and 5 mM NaN_3, similar desensitization occurred, which was again abolished by 20 μM GTP (compare Fig. 1C and 3). In intact cells, desensitization should not occur because the GTP concentration is ~300 μM (Kleinecke et al., 1979), which is 10-fold higher than that required to sustain the IP_3-induced Ca^{2+} release. Indeed, desensitization of the IP_3-induced Ca^{2+} release and its recovery induced by GTP were only observed when permeabilized cells were washed as described in Materials and Methods. We assume that endogenous GTP during this procedure is sufficiently removed, because the effect of added GTP (see Fig. 3) also disappears after the cells are washed.

The mechanism underlying the action of GTP on the IP_3-induced Ca^{2+} release is not understood. An involvement of a "classical" guanine nucleotide-binding protein is rather unlikely because GTPγS could not replace GTP, but rather blocked its effect. In this respect the hydrolysis of GTP seems crucial for its action, which in the classical guanine nucleotide-binding protein terminates the activation. The reversibility of the GTP effect argues against a GTP-catalyzed fusion of intracellular membranes as suggested by Comerford and Dawson (1988). A GTP-driven phosphorylation as observed in liver cells (Dawson et al., 1986) in conjunction with a cellular phosphatase could account for the reversibility of the GTP effect on desensitization reported here. An alternative interpretation of the present data would be that GTP reversibly activates connections between IP_3-sensitive and -insensitive compartments (Ghosh et al., 1989). The action of GTP seems to be not relevant in the tumor cell lines RINA2 (rat insulinoma) or PC12 (rat pheochromocytoma) because these cells showed no decreased response to IP_3 with time (Föhr et al., 1989). In addition, GTP did not increase the IP_3-induced Ca^{2+} release in these cell lines (K. J. Föhr, unpublished data).

Besides GTP, the IP_3-induced Ca^{2+} release also depends on the amount of stored Ca^{2+}. Under the experimental condition shown in Fig. 4A, the IP_3-sensitive Ca^{2+} compartment is only partially filled. The permeabilized cells are still able to take up more Ca^{2+}. This additional Ca^{2+} is directly available for the subsequent Ca^{2+} release. Filling of the compartment (by subsequent additions and uptake of 0.5 nmol of Ca^{2+} by 6.7×10^6 cells) increased the Ca^{2+} release due to 0.1 μM IP_3 (Fig. 4A). After the first addition of 0.5 nmol of Ca^{2+}, the IP_3-induced Ca^{2+} release increased by 84 ± 31.5% (n = 6, mean ± SD). After addition of 1.5 nmol of Ca^{2+}, the IP_3-sensitive Ca^{2+} compartment

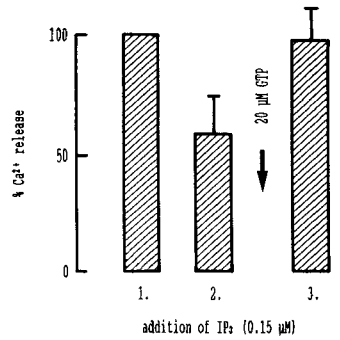

FIG. 3. The effect of GTP on the IP_3-induced Ca^{2+} release is reversible. Cells were first treated as shown in Fig. 1A. Thereafter, they were washed three times as described in Materials and Methods and resuspended in medium containing 150 mM KCl, 20 mM MOPS (pH 7.2), and 5 mM NaN_3. Then the cells took up Ca^{2+} in the presence of Mg^{2+}-ATP, and an almost similar decrease of Ca^{2+} release on repeated IP_3 application was observed (compare with Fig. 1C). The decreased Ca^{2+} release could be abolished by addition of 20 μM GTP. Data are mean ± SD (bars) values (n = 4).

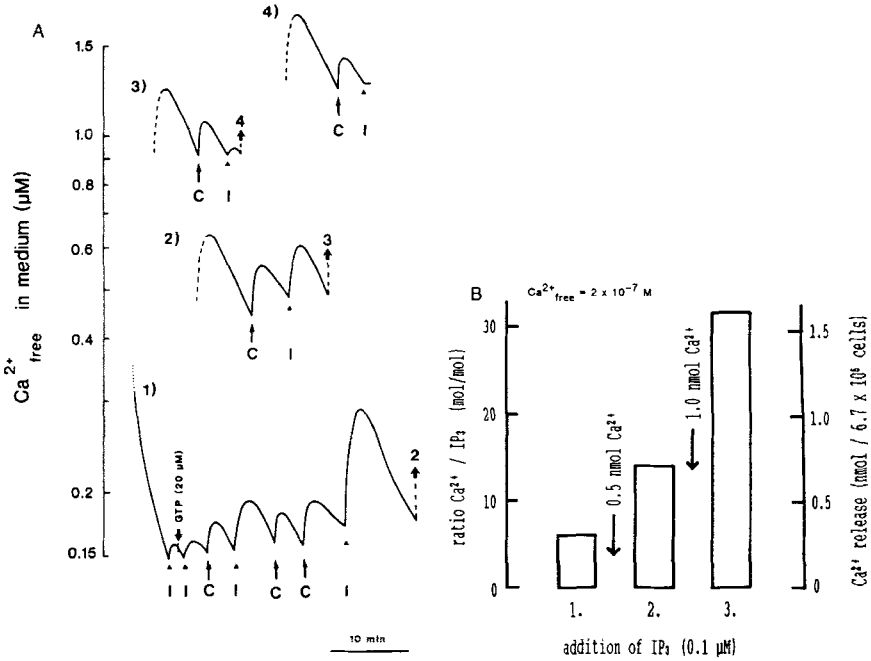

FIG. 4. The Ca^{2+} release is proportional to the amount of stored Ca^{2+} and is inhibited by the medium free Ca^{2+} concentration. **A:** Permeabilized cells (6.7×10^6 cells in a volume of 0.5 ml) were challenged with pulses of 0.1 μM IP_3 (arrowheads). Arrows (C) indicate additions of 0.5 nmol of Ca^{2+}, which was rapidly sequestered. In this way the amount of Ca^{2+} whose release was induced by IP_3 increased. Subsequently the medium free Ca^{2+} concentration was increased by addition of an appropriate amount of Ca^{2+}, and Ca^{2+} release induced by 0.1 μM IP_3 was again determined. **B:** The data shown here are calculated from the experiment shown in A (bottom), which was representative of three similar experiments carried out at 0.2 μM free Ca^{2+}.

is maximally filled because further Ca^{2+} cannot be sequestered. Then, 0.1 μM IP_3 (corresponding to 0.05 nmol of IP_3 in a total volume of 0.5 ml) induced release of 1.6 nmol of Ca^{2+} from 6.7×10^6 cells (Fig. 4). Thus, it can be calculated that one molecule of IP_3 induced the release of up to 30 Ca^{2+} (Fig. 4B). This indicates that the signal can be considerably amplified at the level of the endoplasmic reticulum. A similar ratio between IP_3 and released Ca^{2+} was found using permeabilized RINA2 and PC12 cells (Föhr et al., 1989).

The Ca^{2+} release directly depended on the IP_3 concentration, with a half-maximal Ca^{2+} release at ~0.1 μM IP_3 and a maximal response at 0.25 μM IP_3 (Fig. 5). Maximal IP_3 concentrations induced release of almost 4 nmol of $Ca^{2+}/10^7$ cells (Fig. 5). This is only 10% of the IP_3 concentration necessary to induce release of Ca^{2+} from digitonin-permeabilized chromaffin cells (Stoehr et al., 1986). In addition, the amount of Ca^{2+} released (1.2 nmol/10^7 cells) in the previous report (Stoehr et al., 1986) is only one-third when compared with the 4 nmol of $Ca^{2+}/10^7$ cells found here. One explanation may be that the digitonin-permeabilized cells lose the ability to take up additional Ca^{2+}. This interpretation is supported by the minute amounts of $^{45}Ca^{2+}$ whose release was induced by IP_3 from a nonmitochondrial intracellular compartment in digitonin-permeabilized chromaffin cells (Kao, 1988). In contrast, the present data demonstrate that the IP_3-sensitive Ca^{2+} compartment plays an important role in intracellular Ca^{2+} signaling and control of exocytosis within chromaffin cells.

The IP_3-induced Ca^{2+} release is strongly inhibited by the free Ca^{2+} concentration found in the cytosol of stimulated chromaffin cells (Fig. 4A and 6). To investigate the Ca^{2+} release as a function of the free Ca^{2+} concentration, the Ca^{2+} stores were first completely loaded at the lowest Ca^{2+} concentration investigated (Fig. 4A). GTP (20 μM) was present to avoid desensitization, and Ca^{2+} release was induced with 0.1 μM IP_3. To reach the desired higher free Ca^{2+} concentration, appropriate amounts of Ca^{2+} were added, followed by the next IP_3 application (0.1 μM IP_3). During this stepwise increase of the medium Ca^{2+} concentration, we found that 0.1 μM IP_3 could only induce Ca^{2+} release up to a free medium Ca^{2+} concentration of 1 μM. The inhibitory effect of Ca^{2+} on the IP_3-induced Ca^{2+} release could be overcome by elevating the amount of

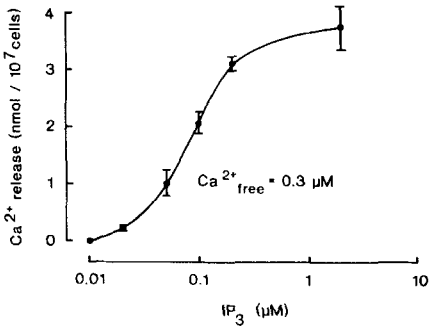

FIG. 5. Ca^{2+} release as a function of the IP_3 concentration. The permeabilized cells were suspended in medium B containing 2 mM Mg^{2+}-ATP. The Ca^{2+} stores were first filled maximally with Ca^{2+} (several additions of Ca^{2+} until no further Ca^{2+} uptake occurred at the free Ca^{2+} concentration indicated). Then 20 μM GTP was added, and IP_3-induced Ca^{2+} release was started. The amount of Ca^{2+} released from 10^7 cells as determined from three experiments carried out at a free Ca^{2+} concentrations of 0.3 μM with 3–6 $\times$ 10^6 cells/400 μl of medium B is shown; data are mean ± SD (bars) values.

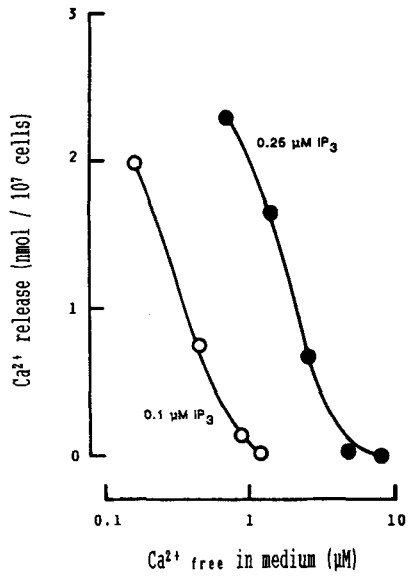

FIG. 6. Ca^{2+} release induced by two different IP_3 concentrations as a function of the free Ca^{2+} concentration. Ca^{2+} stores were first loaded with Ca^{2+} (as described in Fig. 4A). IP_3-induced Ca^{2+} release was examined with 0.1 and 0.25 μM IP_3 in the presence of 20 μM GTP at the free Ca^{2+} concentration in the medium indicated at the abscissa. The experiment with 0.1 μM IP_3 was repeated three times with similar results. No Ca^{2+} release at >1 μM free Ca^{2+} was observed with this IP_3 concentration (see also Fig. 4A).

IP_3 (here 0.25 μM; Fig. 6). However, at free Ca^{2+} concentrations of >8 μM no further release of Ca^{2+} could be induced, even when higher IP_3 concentrations were applied. Thus, in endocrine cells a negative feedback control of the IP_3-induced Ca^{2+} release by Ca^{2+} itself exists. It is noteworthy that a release of 4 nmol of Ca^{2+} within 10^7 chromaffin cells [assuming a cell volume of 6 $\mu l/10^7$ cells as reported by Hampton and Holz (1983) and disregarding Ca^{2+} buffering by the cells] would yield an intracellular free Ca^{2+} concentration of >600 μM. Inhibition of IP_3-induced Ca^{2+} release by Ca^{2+} as documented in Fig. 6 are among the mechanisms that assure that such high and probably poisonous Ca^{2+} concentrations are not reached within the cells by IP_3-induced Ca^{2+} release from intracellular compartments. Indeed, stimulation of nonpermeabilized chromaffin cells with various stimuli led to an increase of the intracellular free Ca^{2+} concentration between 200 and 400 nM above basal values (Cheek et al., 1989). An inhibitory effect of Ca^{2+} on IP_3-induced Ca^{2+} release has also been found in other permeabilized cell preparations (Hirata et al., 1984; Chueh and Gill, 1986; Joseph et al., 1989), which may be a consequence of the recently discovered inhibition of IP_3 binding to its receptor protein (Worley et al., 1987; Danoff et al., 1988; Supattapone et al., 1988). On the other hand, IP_3-induced Ca^{2+} release from thrombocytes is insensitive to 1–100 μM free Ca^{2+} (Brass and Joseph, 1985).

In summary, we have demonstrated that adrenal chromaffin cells, in contrast to earlier reports (Stoehr et al., 1986; Kao, 1988), exhibit a highly active IP_3-induced Ca^{2+} uptake system from which release of Ca^{2+} can be induced by low levels of IP_3. The release is immediately followed by Ca^{2+} resequestration, and GTP assures full activity during repetitive stimulation. It can thus be concluded that the Ca^{2+} release from intracellular stores of chromaffin cells yields enough Ca^{2+} for the activation of intracellular processes such as exocytosis.

Acknowledgment: This work was supported by the Deutsche Forschungsgemeinschaft (Gr 681). For technical help in the construction and use of the Ca^{2+}-specific electrode we are indebted to S. Galler (Konstanz, F.R.G.). The technical assistance of Mrs. M. Rudolf is gratefully acknowledged.

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