Thyrotropin-releasing hormone-mediated Mn^{2+} entry in perfused rat anterior pituitary cells

Zong Jie CUI and Priscilla S. DANNIES*

Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510, U.S.A.

Receptor-mediated Ca^{2+} influx has been shown to exist in several cell types. Thyrotropin-releasing-hormone (TRH)-stimulated Ca^{2+} entry has also been postulated to exist in rat anterior pituitary cells, but direct evidence has been lacking. We have measured the fluorescence quenching of indo-1 caused by Mn^{2+} at a Ca^{2+} -insensitive wavelength to investigate the actions of TRH on cation entry in dispersed perfused anterior pituitary cells. In indo-1-loaded cells perfused with Ca^{2+} -free medium, Mn^{2+} caused fluorescence quenching in unstimulated cells; TRH caused further quenching. TRH-stimulated Mn^{2+} entry was transient, and levelled off within a few minutes in the presence of continuous TRH infusion. TRH-stimulated Mn^{2+} entry was dependent on the concentration of Mn^{2+} (50 μM –1 mM). TRH (1 μM) caused a larger effect than TRH (10 nM). La^{3+} and Ni^{2+} blocked the quenching stimulated by TRH. The rate of basal quenching was not blocked by dopamine, but TRH-stimulated Mn^{2+} entry was partially blocked by 1 μM -dopamine and almost completely abolished by 10 μM -dopamine. Thapsigargin (1–5 μM), a tumour promotor which depleted intracellular Ca^{2+} stores, had little effect on Mn^{2+} . F^{-} (20 mM), which activates G-proteins, also had little effect on Mn^{2+} entry. We conclude that TRH can transiently stimulate Ca^{2+} entry through a channel than can pass Mn^{2+} and be inhibited by dopamine. Depleting Ca^{2+} stores alone is not sufficient to stimulate Ca^{2+} entry, and so TRH must do so by other mechanisms.

INTRODUCTION

The understanding of Ca^{2+} entry in anterior pituitary cells has greatly advanced since action potentials with a component that depended on Ca^{2+} were first observed in these cells (Kidokoro, 1975; Taraskevich & Douglas, 1977, 1980; Ozawa & Sand, 1978). We now know that there are two types of voltage-dependent Ca^{2+} channels in anterior pituitary cells: the high-threshold and slowly inactivating L-type, and the low-threshold and rapidly inactivating T-type. They are well characterized electrophysiologically, and channel activation causes direct hormone secretion (Mattheson & Armstrong, 1986; Cohen & McCarthy, 1987; Enyeart *et al.*, 1987; Izumi *et al.*, 1989, 1990).

Stimulation of lactotrophs and pituitary tumour cells by thyrotropin-releasing hormone (TRH) causes intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) to increase in a biphasic fashion, with a transient spike followed by a plateau phase (Albert & Tashjian, 1984b; Gershengorn, 1986; Drummond, 1986; Law *et al.*, 1989). The transient spike is caused, at least in part, by mobilization of intracellular stores and is at least partially independent of extracellular Ca^{2+} . The second long-lasting phase is dependent on extracellular Ca^{2+} and independent of mobilization of $Ins-(1,4,5)P_3$ (Gershengorn, 1986; Drummond, 1986; Mollard *et al.*, 1990). The long-lasting phase is not adequately explained, although it sometimes has been assumed to be caused by entry through voltage-dependent Ca^{2+} channels, on the basis of pharmacological evidence using Ca^{2+} -channel blockers. This evidence is not conclusive. Experiments using these drugs in fact do not completely block the sustained rise in Ca^{2+} (Albert & Tashjian, 1984a,b; Wood & Schofield, 1989; but see Suzuki *et al.*, 1990). Direct measurements of Ca^{2+} -channel activity in pituitary tumour cells by electrophysiological techniques indicate that TRH actually inhibits L-type channel activity and does not affect T-channels (Simasko, 1991; Kramer *et al.*, 1991). In addition, attempts to detect TRH stimulation of initial rates of Ca^{2+} entry

by measuring $^{45}Ca^{2+}$ uptake have not shown such stimulation (Pachter *et al.*, 1988). To demonstrate that TRH-induced Ca^{2+} entry does exist, we used a different approach. We have investigated whether TRH can directly stimulate Ca^{2+} entry by measuring the Mn^{2+} quench of the fluorescence of intracellularly loaded indo-1.

Receptor-mediated Ca^{2+} entry through channels other than voltage-dependent ones has been well documented in a number of cell types, including leucocytes, neutrophils, platelets, endothelial cells, myocytes, pancreatic acinar cells, lacrimal acinar cells and hepatocytes (Merritt & Hallam, 1988, 1989; Sage *et al.*, 1989; Muallem *et al.*, 1990; Jacob, 1990; Chien *et al.*, 1990; Crofts & Barritt, 1990; Kass *et al.*, 1990; Mertz *et al.*, 1990; Kwan & Putney, 1990), by using this ability of Mn^{2+} to quench intracellular fluorescent indicators. By substituting extracellular Mn^{2+} for extracellular Ca^{2+} and measuring the fluorescence of intracellular indo-1 at a wavelength insensitive to changes in $[Ca^{2+}]_i$, we have been able to demonstrate that TRH causes receptor-mediated Ca^{2+} entry in freshly isolated anterior pituitary cells.

MATERIALS AND METHODS

Anterior pituitary cells were dispersed by sequential digestion with trypsin and viokase (Pancreatin; Sigma) as described previously (Law *et al.*, 1988). Briefly, female retired breeders of the Sprague–Dawley strain rats were killed by decapitation, and the anterior pituitary gland was taken out and cut into 1 mm³ pieces, which were then incubated in bivalent-cation-free medium containing trypsin (1 mg/ml) at 37 °C. Digestion was terminated 30 min later by trypsin inhibitor (1 mg/ml) in bivalent-cation-containing medium, and the pieces were subsequently incubated in phosphate buffer with viokase (1 mg/ml) for 90 min (37 °C). At the end of this digestion, cells were dispersed by repeatedly passing through a Pasteur pipette, and then were layered on to

Abbreviations used: TRH, thyrotropin-releasing hormone; $[Ca^{2+}]_i$, concentration of intracellular free Ca^{2+} ; DTPA, diethylenetriaminepenta-acetic acid.

* To whom correspondence should be addressed.

Fig. 1. Emission spectra of indo-1

Indo-1 free acid (1 mM in dimethyl sulphoxide; 2 μ l) was added to 2 ml of buffer (KCl 140 mM, EGTA 1 mM, Hepes 10 mM, pH 7.3) and the emission spectrum before (A) and after (B) further addition of cation (2 mM) was recorded: (a) Ca^{2+} ; (b) La^{3+} ; (c) Ni^{2+} ; (d) Mn^{2+} .

BSA (4 %, w/v) and centrifuged at 400 g for 2 min, washed once with the basic buffer and resuspended in the same buffer (4 ml). Indo-1 acetoxymethyl ester (AM) (10 μ M) was added, and loading was carried out for 30 min at 37 $^{\circ}\text{C}$.

Cells loaded with indo-1 were pelleted and resuspended in the basic buffer, placed into a quartz flow cuvette [(1–2) $\times 10^6$ cells] and aligned with Bio-Gel beads (P2) to the light path of the fluorimeter and perfused at 0.6 ml/min. Fluorescence was measured with an excitation wavelength of 350 nm and different emission wavelengths in an SLM 8000C spectrofluorimeter. For time-course studies the fluorescence was normalized to that at 1 s, which was set at 1.0 or occasionally 0.95 for clarity (Figs. 2–8). The band widths for excitation and emission were 4 nm and 8 nm respectively. Two cuvettes were usually prepared at the same time and used for each experiment. The order in which the cuvettes were used did not affect the response.

The basic buffer that we used has the following composition (in mM): NaCl 120, KCl 4.8, KH_2PO_4 1.2, NaHCO_3 20, CaCl_2

Fig. 2. TRH-stimulated Mn^{2+} influx in pituitary cells is dependent on extracellular $[\text{Mn}^{2+}]$

Indo-1-loaded cells were perfused and Mn^{2+} [100 μ M (a) or 200 μ M (b)] and TRH (1 μ M) (a, b) were added as indicated. Fluorescence was recorded at 430 nm. Similar results were observed in two experiments with 100 μ M extracellular Mn^{2+} and in three other experiments with 200 μ M Mn^{2+} .

1.8, MgSO_4 1.0, glucose 5.0, Hepes 10.0, BSA 1 mg/ml, pH adjusted to 7.35 with NaOH. For Ca^{2+} -insensitive wavelength determination experiments, the basic buffer was used; for all other experiments involving fluorescence quenching with Mn^{2+} , Ni^{2+} or La^{3+} , the buffer composition was as follows (in mM): NaCl 120, KCl 4.8, MgCl_2 1.0, glucose 5.0, Hepes 10.0, pH adjusted to 7.35 with NaOH.

We had previously shown that repetitive pulses of TRH each caused a similar increase in $[\text{Ca}^{2+}]_i$ (Law *et al.*, 1989), and therefore we used this procedure to determine the Ca^{2+} -insensitive wavelength of indo-1 in intact cells. We challenged the perfused cells with 10 s pulses of TRH (0.1 μ M) at 11 min intervals, and confirmed that in these serum-free conditions we still saw a change in $[\text{Ca}^{2+}]_i$ at each pulse (results not shown). Then we measured the change in fluorescence after each pulse at various wavelengths. Indo-1 fluorescence did not change at 430 nm when $[\text{Ca}^{2+}]_i$ rose. Fluorescence decreased or increased with each TRH spike at wavelengths greater or smaller than 430 nm respectively. Quenching experiments were therefore performed at the emission wavelength of 430 nm.

Indo-1 AM and the free acid of indo-1 were from Molecular Probes (Eugene, OR, U.S.A.). TRH was a gift from Abbott Laboratories (North Chicago, IL, U.S.A.). Thapsigargin was from LC Services (Woburn, MA, U.S.A.).

RESULTS

Data in Fig. 1 illustrate the effects of inorganic cations on fluorescence of indo-1 free acid in solution. In a high- K^+ buffer (KCl 140 mM, Hepes 10 mM, pH 7.35), the free acid has an emission of 472 nm that shifted to about 398 nm upon addition of cations. It is noteworthy that the isosbestic point of indo-1 for different cations is different. We found it to be 447 nm, 441 nm and 395 nm for Ca^{2+} , La^{2+} and Mn^{2+} respectively; that for Ni^{2+} could not be determined. These spectra confirm that, when fluorescence is measured at a wavelength insensitive to Ca^{2+} (the isosbestic point), it is still sensitive to Mn^{2+} . We examined the rate of bleaching of indo-1 free acid in the same solution at 430 nm and found the rates were similar whether indo-1 was uncomplexed or complexed to the different cations Mn^{2+} , Ni^{2+} and La^{2+} (results not shown). As described in the Materials and methods section, we determined the Ca^{2+} -insensitive emission wavelength to indo-1 in intact cells; the wavelength is shifted to 430 nm, which is apparently due to a blue-shift of indo-1 free acid emission spectrum in cytoplasm (Owen & Shuler, 1989; see the Discussion section).

We determined if TRH stimulated influx of bivalent cations from the extracellular space by perfusing cells with a buffer to which no Ca^{2+} was added and then adding Mn^{2+} and TRH and

Fig. 3. Effects of TRH concentration on Mn^{2+} influx

Indo-1 loaded cells were perfused and Mn^{2+} (1 mM; a b) and TRH [10 nM (a) or 1000 nM (b)] were added as indicated. Fluorescence was recorded at 430 nm. Similar results were observed with 10 nM-TRH in two other experiments and with 1000 nM-TRH in ten other experiments.

Fig. 4. Effects of Ni^{2+} , La^{3+} and Mn^{2+} on indo-1 fluorescence in unstimulated cells: reversal by removal of the cation from the perfusion medium or by chelation with DTPA

La^{3+} (1 mM; trace a), Ni^{2+} (1 mM; trace b) or Mn^{2+} (1 mM; trace c) and DTPA (2 mM) were added as indicated. Fluorescence was recorded at 430 nm. Similar traces were obtained in two or three other independent experiments. MCl_n indicates the chloride salt of the n -valency cation M.

measuring fluorescence emitted at the Ca^{2+} -insensitive wavelength. As shown in Fig. 2, there was a gradual decline in fluorescence before the addition of Mn^{2+} (part 1 of the traces). This gradual decline occurred, but to a lesser extent, in perfused cells that were not continuously exposed to excitation light, indicating about half of the decline is caused by loss of indo-1 from the cells. The other half of the loss is a result of bleaching of the dye. When Mn^{2+} was added, there was a rapid fall in the fluorescence, followed by a slower rate of fluorescence loss that was faster than that detected in the absence of Mn^{2+} (parts 2 and 3 of the traces in Fig. 2). Most significantly, when TRH was added, there was another rapid fall in fluorescence which levelled off gradually (parts 4 and 5 of the traces in Fig. 2). These results indicate that TRH can stimulate Mn^{2+} entry into the perfused anterior pituitary cells to cause quenching of the fluorescence of indo-1 in the cytoplasm. The TRH-quenching effect was dependent on extracellular $[Mn^{2+}]$ (Fig. 2), consistent with the hypothesis that TRH opens channels in the plasma membrane that allow Mn^{2+} to cross freely. We also detected an enhanced rate of quench when we depolarized the cells with high K^+ concentrations or stimulated Ca^{2+} channels with 1 μ M-BAY K 86744, indicating that Mn^{2+} can pass through voltage-dependent Ca^{2+} channels (results not shown). After TRH-stimulated quench of indo-1 fluorescence, we obtained further quench by adding ionomycin; therefore not all the indo-1 loaded has been quenched by TRH (1 μ M).

Unlike other cell types that have been used for Mn^{2+} -quench studies, the basal entry of Mn^{2+} is always greater than that stimulated by TRH, so that quantitative analysis of TRH-

Fig. 5. Ni^{2+} blockade of TRH-stimulated Mn^{2+} influx

Indo-1-loaded pituitary cells were perfused and Ni^{2+} [0 (a) or 1 mM (b)], Mn^{2+} (1 mM; a, b) and TRH (1 μM ; a, b) were added as shown. Fluorescence was recorded at 430 nm. Similar results were observed in two other experiments.

Fig. 6. La^{3+} blockade of TRH-stimulated Mn^{2+} influx

Indo-1-loaded pituitary cells were perfused and La^{3+} [0 (a) or 1 mM (b)], Mn^{2+} (1 mM; a, b) and TRH (1 μM ; a, b) were added as indicated. Fluorescence was recorded at 430 nm. Similar results were observed in two other independent experiments.

stimulated entry was not attempted. However, when we compared two concentrations of TRH in the same experiment, the effect of 1 μM was much larger than that of 10 nM (Fig. 3). In other experiments, we demonstrated that the effect of 100 nM-TRH is similar in magnitude to that detected with 1 μM .

The initial rapid fall in fluorescence (Fig. 2, part 2 of the trace) appears to be caused by quenching of an indo-1 pool accessible to extracellular medium. Evidence for this pool is that this rapid quenching is just as rapidly reversed when Mn^{2+} is removed from the perfusion medium or when the cell-impermeant chelator diethylenetriaminepenta-acetic acid (DTPA) is added to the perfusion medium (Fig. 4). We also investigated the effects of Ni^{2+} and La^{3+} , which have been shown to prevent Ca^{2+} entry without entering cells in other systems (Kwan and Putney, 1990; Crofts & Barritt, 1990; Kass *et al.*, 1990). Ni^{2+} caused an immediate decrease in fluorescence when added to the perfusion medium, and the effect reversed rapidly with the addition of DTPA to or removal of Ni^{2+} from the perfusion medium (Fig. 4). La^{3+} also caused a rapid effect on indo-1 fluorescence, in this case an increase (Fig. 4). This effect was not rapidly reversed when La^{3+} was removed from the perfusion medium, but was rapidly reversed when cells were perfused with DTPA (Fig. 4). La^{3+} did not stabilize the rate of indo-1 bleach in solution; the stabilization of the fluorescence in the cells may be caused by decreased leakage from the cells in the presence of La^{3+} . We found that

TRH had no effect on fluorescence in the presence of Ni^{2+} or La^{3+} (without Mn^{2+} ; results not shown). When Mn^{2+} alone was present, the rate of basal quench after the rapid fall was enhanced (Fig. 2, compare parts 1 and 3). This enhanced rate did not occur in the presence of Ni^{2+} alone (Fig. 5). As shown in Figs. 2 and 3, TRH can stimulate Mn^{2+} entry, but, when Ni^{2+} or La^{3+} was present in the perfusion medium, TRH no longer stimulated Mn^{2+} quench of indo-1 fluorescence (Figs. 5 and 6).

It has been shown previously that dopamine decreased TRH-induced prolactin release without interfering with the mobilization of intracellular Ca^{2+} (Law *et al.*, 1988). In the present experiment (Fig. 7), dopamine (10 μM) was added 4 min before the addition of Mn^{2+} ; it did not affect initial Mn^{2+} quenching of indo-1, but it almost completely abolished TRH-stimulated Mn^{2+} influx. Therefore dopamine appears to inhibit only TRH-stimulated Mn^{2+} entry, but not basal entry. The G-protein stimulator F^- at 20 mM had little effect on the rate of Mn^{2+} -induced decrease in fluorescence (Fig. 8).

Thapsigargin has been shown to deplete intracellular Ca^{2+} stores in a number of cell types; it depletes TRH-releasable stores in pituitary tumour cells with a time scale and efficacy similar to TRH-stimulated Ca^{2+} release (Law *et al.*, 1990). We found, however, that thapsigargin had little or no effect on Mn^{2+} entry. To demonstrate that thapsigargin was stimulating release of Ca^{2+} from these cells at a time when it had little effect on Mn^{2+} entry,

Fig. 7. Dopamine blockade of TRH-stimulated Mn^{2+} influx

Indo-1-loaded pituitary cells were perfused and dopamine [0 (a) or 10 μM (b)], Mn^{2+} (1 mM; a, b) and TRH (1 μM ; a, b) were added as shown. Fluorescence was recorded at 430 nm. Similar results were observed in two other experiments.

Fig. 8. F^- did not induce Mn^{2+} influx

Indo-1-loaded pituitary cells were perfused and Mn^{2+} (1 mM; a, b) and TRH (1 μM ; a) or F^- (20 mM; b) were added as shown. Fluorescence was recorded at 430 nm. Both traces a and b were reproducible in three other experiments.

we used a fluorimeter in which we could simultaneously measure a Ca^{2+} -sensitive emission wavelength as well as a Ca^{2+} -insensitive one, and showed that thapsigargin does cause an increase in $[Ca^{2+}]_i$ (Fig. 9).

DISCUSSION

The spectral data in Fig. 1 confirm that indo-1 free acid in a buffer solution has an emission maximum of 472 nm, and the maximum shifts to the blue region by 74 nm on addition of Ca^{2+} , La^{2+} , Ni^{2+} and Mn^{2+} (to 398 nm); fluorescence is decreased in the presence of Ni^{2+} and Mn^{2+} . These data directly validated the experimental approach of using Mn^{2+} quenching of indo-1 fluorescence to monitor the opening of bivalent-cation channels. We did not determine the Ca^{2+} isosbestic point of indo-1 *in situ* because it would be difficult to clamp intracellular Ca^{2+} concentrations for the time period necessary to obtain an emission spectrum without disrupting the cell plasma membrane. Instead we measured the indo-1 fluorescence changes at different wavelengths after TRH application. The Ca^{2+} -insensitive wavelength that we determined is 17 nm less than the isosbestic point found in cell-free solutions. This blue shift is not surprising, because it has been shown that the emission maximum of intracellular indo-1 has a blue shift in comparison with indo-1 free acid in aqueous solution; e.g. the emission maximum is 470 nm in

aqueous solution and 450 nm in cells (Owen & Shuler, 1989). The intracellular isosbestic point of indo-1 should also have a blue shift, because the intracellular milieu does not change the emission spectrum of Ca^{2+} -bound indo-1 (Owen & Shuler, 1989).

We found that TRH can stimulate Mn^{2+} quenching of indo-1, but does not affect indo-1 fluorescence when Ni^{2+} or La^{3+} alone is present in the perfusion medium (results not shown). This may be an indication that there is an indo-1 pool that can only be entered by Mn^{2+} . We have also found that all three cations have rapid effects on indo-1 fluorescence in unstimulated cells. Other groups have found such rapid effects of Mn^{2+} and Ni^{2+} in hepatocytes with fura-2 and quin-2 and have demonstrated that the effects are caused by extracellular fluorescent indicators, because the effects are rapidly reversed by the extracellular chelator DTPA (Kass *et al.*, 1990; Crofts & Barritt, 1990). These investigators suggest the indicator is in the medium. We can recover the fluorescence rapidly with the chelator DTPA, but, since we are using a perfusion system, fluorescent indicators not bound to the cells will be rapidly washed away (demonstrated by the removal of Ni^{2+} ; see Fig 4). We interpret our results to indicate that there is a compartment of indo-1 associated with the cells, perhaps in the membrane, that is accessible from extracellular space and is responsible for the rapid effect. Such a pool of indo-1 will clearly contribute to the fluorescence signal when Ca^{2+} is measured, because in normal Ca^{2+} -containing medium it will be complexed with extracellular Ca^{2+} . When

measurements have been made in pituitary cells by using indo-1 in the absence of Ca^{2+} , basal $[\text{Ca}^{2+}]_i$ decreased immediately to a new steady state (Law *et al.*, 1988; Kuan *et al.*, 1990; Login *et al.*, 1990), consistent with a compartment of indo-1 that is sensitive to extracellular Ca^{2+} . Therefore there seem to be at least two indo-1 pools in perfused anterior pituitary cells: one that is readily accessible to Ni^{2+} , La^{3+} , Mn^{2+} , or even to EGTA (see Kuan *et al.*, 1990; Login *et al.*, 1990), and another one that can be entered by Mn^{2+} only when stimulated by TRH.

As discussed in the Introduction, there had not been a direct demonstration of TRH-stimulated bivalent-cation entry before this present study, although such entry had been postulated and sometimes assumed to exist. We have now shown that TRH stimulates quench of indo-1 fluorescence by Mn^{2+} , and therefore must stimulate Mn^{2+} entry. Ca^{2+} entry through these channels may contribute to the increase in $[\text{Ca}^{2+}]_i$ caused by TRH. Suzuki *et al.* (1990) found that Ni^{2+} blocks the sustained increase in $[\text{Ca}^{2+}]_i$ caused by TRH in GH₃ cells, indicating that entry that is blocked by Ni^{2+} plays a role in the sustained increase. The TRH-stimulated Mn^{2+} entry has this property.

How TRH stimulates Mn^{2+} entry is not clear. It has been proposed that depletion of Ca^{2+} stores causes Ca^{2+} entry, but, as was found in hepatocytes (Kass *et al.*, 1990; Llopis *et al.*, 1991), depletion of inositol-sensitive stores does not appear to have a major effect in pituitary cells, because thapsigargin had little or no effect on Mn^{2+} entry, although it has been reported to do so in endothelial cells, pancreatic acinar cells, neutrophils and a T-cell line (Jacob, 1990; Mertz *et al.*, 1990; Llopis *et al.*, 1991; Montero *et al.*, 1991). Most of the TRH stimulation of Mn^{2+} entry therefore must be caused by an action directly on a channel or through actions of second messengers. One second messenger produced by TRH is diacylglycerol, which activates protein kinase C (Martin *et al.*, 1990). Marchetti & Brown (1988) and Tornquist & Tashjian (1990) suggests that both L- and T-type Ca^{2+} channels may be inactivated by the protein kinase C activators 1-oleoyl-2-acetyl-sn-glycerol or phorbol 12-myristate 13-acetate. More recent results indicate that TRH inhibits L-channels but does not affect T-channels in pituitary tumour cells (Simasko, 1991; Kramer *et al.*, 1991). These results suggest that TRH does not activate L- and T-type channels, and therefore Ca^{2+} (Mn^{2+}) entry has to occur through channels not previously described in pituitary cells, i.e. receptor-operated Ca^{2+} channels.

If TRH coupled directly to a channel, it could do so through G-proteins. The TRH receptor is coupled to phosphatidylinositol hydrolysis by a G-protein other than G_s and G_i (Aub *et al.*, 1986; Wojcikiewicz *et al.*, 1986), and dopamine exerts its inhibitory effects on TRH via the G_i protein (Ohmichi *et al.*, 1990). Therefore the lack of effect of F^- does not necessarily rule out an effect occurring through G-proteins, because the activating effect of F^- is not specific and all G-proteins are equally susceptible. The presence of F^- may be analogous to the simultaneous presence of TRH and dopamine (Fig. 8).

The inhibitory effects of dopamine on initial Mn^{2+} quenching of indo-1 are not obvious in our experiments, which is consistent with the $^{45}\text{Ca}^{2+}$ -flux work by Tam & Dannies (1980) but different from the work of Lafond & Collu (1986) and of Login *et al.* (1988). The lack of inhibitory effect of dopamine on basal Mn^{2+} entry in our present work may be caused by the short incubation period before the addition of Mn^{2+} . TRH-stimulated Mn^{2+} entry can be completely blocked by dopamine, indicating that TRH-activated Mn^{2+} entry may have different properties from the basal Mn^{2+} entry process.

In summary, we have directly observed TRH-stimulated Mn^{2+} (Ca^{2+}) entry in perfused isolated anterior pituitary cells and found this process does not occur when intracellular Ca^{2+} stores are depleted. Functionally, this receptor-mediated Ca^{2+} entry

Fig. 9. Lack of effect of thapsigargin (Tg) on Mn^{2+} entry

In order to monitor fluorescence at two wavelengths at the same time, these experiments were done with an SLM 4800S spectrofluorimeter. By the same process described in the Materials and methods section, it was found that the Ca^{2+} -insensitive wavelength of indo-1 *in situ* in the SLM 4800S instrument was 412 nm. Trace a (upper part, 390 nm) shows that Tg (1 μM) increased $[\text{Ca}^{2+}]_i$, but Mn^{2+} entry was undetectable (lower part, 412 nm). A pulse (1 min) of ionomycin (1 μM) induced quenching of fluorescence at both 412 nm (F_{412}) and 390 nm (F_{390}). Trace b is a separate recording of the ratio of fluorescence (F_{390}/F_{475}) under conditions identical with those for trace a, except for the absence of added extracellular Mn^{2+} ; it is clear that both Tg (1 μM) and ionomycin (1 μM) caused $[\text{Ca}^{2+}]_i$ to increase. Mn^{2+} (1 mM), Tg (1 μM) and ionomycin (1 μM , 1 min) were added as indicated. We obtained similar results in three other experiments.

may be important to maintain refractoriness of prolactin secretion that develops after repeated stimulation with TRH (Law *et al.*, 1989).

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