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Verapamil: Influence upon Basal and Stimulated Rat Growth Hormone and Prolactin Release *in Vitro*[1] (41617)

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Abstract. Verapamil is an organic calcium antagonist which is believed to prevent the passage of calcium (Ca^{2+}) across the cell membrane into the cell. In a rat pituitary perifusion–immunoprecipitation system, verapamil (50 μM) prevents the inhibitory effect of increased extracellular Ca^{2+} (5.4 mM) on basal and stimulated release of stored, prelabeled [^{3}H]GH and [^{3}H]PRL. [^{3}H]GH release from pituitary explants perifused in standard medium (GIBCO Minimum Essential Medium: 1.8 mM Ca^{2+}) is transiently increased by 50 μM verapamil while [^{3}H]PRL release is suppressed. With continued exposure to 50 μM verapamil, [^{3}H]GH release rates fall below (89.8 ± 2.1% of base) preverapamil levels while [^{3}H]PRL release rates simply remain suppressed (48.2 ± 7.3% of base). With 250 μM verapamil, poststimulatory inhibition of [^{3}H]GH release occurs more quickly, and after its withdrawal rebound release of both GH and PRL occur. Inhibition of [^{3}H]GH release by 25 nM somatostatin (SRIF) and post-SRIF rebound [^{3}H]GH release is not prevented by 50 μM verapamil. The early, rapid [^{3}H]GH release phase of 1 mM dibutyryl cyclic AMP (dbcAMP) stimulation is potentiated by verapamil pretreatment, but only if the verapamil is continued during dbcAMP stimulation. Potassium (21 mM K^+)-stimulated release of both ^{3}H-labeled hormones is inhibited after similar pretreatment 50 μM verapamil. Conclusions: (a) verapamil antagonizes the inhibitory effects of increased extracellular Ca^{2+} on basal or dbcAMP-stimulated [^{3}H]GH and [^{3}H]PRL release; (b) in standard medium (1.8 mM Ca^{2+}), 50 μM verapamil increases basal [^{3}H]GH release suggesting either a direct effect or an antagonism of 1.8 mM extracellular Ca^{2+}; (c) although verapamil-sensitive Ca^{2+} movement is not necessary for dbcAMP stimulation of [^{3}H]GH release, verapamil potentiates dbcAMP-stimulated release; (d) because verapamil also inhibits K^+-stimulated [^{3}H]GH and [^{3}H]PRL release, these observations support previous suggestions that K^+- and dbcAMP-stimulated rapid hormone release occurs from different intracellular sites; and (e) because verapamil does not prevent any phase of SRIF action and since these two agents differentially alter K^+- and cAMP-stimulated release, their mechanisms of action must partially differ.

Calcium (Ca^{2+}) is an important factor in anterior pituitary hormone release mechanisms, although its role is not yet precisely defined. Decreasing medium Ca^{2+} can prevent the release of all pituitary hormones (1–4) in response to a variety of secretagogues; release of these hormones may result from extracellular Ca^{2+} influx according to the stimulus–secretion coupling concept (5). It also has been suggested that the action of hypothalamic releasing hormones on the pituitary is mediated by increases in cyclic 3′,5′-AMP (6). Cyclic AMP-mediated growth hormone (GH) release *in vitro* requires Ca^{2+} (7), and the removal of Ca^{2+} prevents that release (8). However, while decreased medium Ca^{2+} inhibits hypothalamic extract-stimulated hormone release, simultaneous stimulation of intrapituitary cyclic AMP is not prevented (9). Thus, if cAMP levels and hormone release are coupled, perhaps through Ca^{2+}, the effect of decreased Ca^{2+} must occur late in the secretory process. Such coupling has been challenged for GH (10), prolactin (PRL) (11), and in fact the release of gonadotropin has been dissociated from changes in cAMP but tied to changes in intracellular Ca^{2+} levels alone (12).

Verapamil is an organic Ca^{2+} antagonist which prevents influx of Ca^{2+} across the cell

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membrane (13). In myocardium it may specifically inhibit processes involved in the coupling of excitation and contraction (14, 15) analogous to stimulus–secretion coupling (5). It interferes directly with the Ca^{2+} binding sites of ionophores including, presumably, native membrane-associated Ca^{2+} binding–transport systems (16). Verapamil has been shown to prevent Ca^{2+} effects on islet (17) and pituitary (18, 19) hormone release. In the pituitary it is reported to not inhibit basal GH release but to inhibit high K^+ and ouabain-induced GH release (18) through prevention of Ca^{2+} entry, but not intracellular redistribution (19).

Increased extracellular Ca^{2+} (5.4 m*M*) can itself inhibit GH release *in vitro* (20), in contrast to its ability in the pancreatic islet to reverse somatostatin (SRIF) inhibition of stimulated hormone release (21). The experiments reported here were undertaken in an attempt to specifically prevent with verapamil the effects of increased extracellular Ca^{2+} on GH release and thus to validate the effect of increased extracellular Ca^{2+}. Further, verapamil's effects in the presence of more physiologic concentrations of Ca^{2+} (1.8 m*M*) were examined to provide information concerning the role of ambient Ca^{2+} in the release process. The perifusion–immunoprecipitation system was chosen for these studies in order to (a) limit observations to release of stored hormone and eliminate the contribution of newly synthesized hormone, (b) permit sequential medium alteration without manipulation of pituitary explants, and (c) permit study of the time course of hormone release responses.

Materials and Methods. *Perifusion of prelabeled pituitaries.* In each experiment, anterior pituitary quarters from three male Holtzman rats (180–250 gm) were preincubated for 150 min in (1.8 m*M* Ca^{2+}) Minimum Essential Medium (GIBCO) supplemented with 10 mg/ml bovine serum albumin and 10 μCi/ml [^{3}H]leucine (SA 32 Ci/mmole, New England Nuclear) in order to homogeneously prelabel stored GH ([^{3}H]GH) and PRL ([^{3}H]PRL) (22). They were then perifused (23) in MEM without [^{3}H]leucine permitting continued synthesis of unlabeled hormone, but not of labeled hormone (23). Thus, by studying release of prelabeled specific immune precipitable isotope, hormone release from intracellular storage may be examined. Independent effects upon hormone synthesis and release of newly synthesized hormone are thereby eliminated. Such precision in the definition of the source of released hormone is not possible using radioimmunoassay which could not distinguish between newly synthesized and stored hormone.

Perifusion media were supplemented with verapamil (Knoll Pharmaceutical), Ca^{2+}, SRIF, and/or dbcAMP in various combinations and concentrations as described in the Results. Release of ^{3}H-labeled hormone is not altered by the mechanics of medium change in this system (22). The pH of all media was adjusted to 7.2–7.3, and flow was maintained at 0.2 ml/min. Effluent fractions were collected at 5-min intervals. At the conclusion of each experiment, pituitaries were homogenized in 1 ml 0.01 *N* NaOH at 4°C with a 1-ml saline wash, centrifuged at 12,000*g* for 10 min, and the supernate recovered.

Immunoprecipitation of rGH and rPRL. Aliquots of perifusion effluent and pituitary homogenate were assessed for [^{3}H]GH and [^{3}H]PRL by specific double antibody immunoprecipitation (IP) techniques using rGH- or rPRL-specific antisera raised in baboons and standardized against antisera prepared in a rhesus monkey (24), a rabbit (25), and NIAMD-anti-rat GH serum-4 or anti-rat PRL serum-6. Antiserum sufficient to precipitate all the GH or PRL in each aliquot was used and the soluble hormone–antibody complex was precipitated by goat anti-monkey gamma globulin serum. For each sample, nonspecific absorption of radioactivity to the immune complex was assessed by a parallel IP in which normal baboon serum substituted for specific antiserum. Specific ^{3}H-labeled hormone is the difference between antiserum- and normal serum-precipitated radioactivity (As/NlS = 2.5 − 80/1).

Calculation of ^{3}H-labeled hormone release. The ^{3}H-labeled hormone release rate is expressed as percentage per minute of the ^{3}H-labeled hormone remaining in the pituitary at the onset of the particular collection interval. The latter is calculated by subtracting specific ^{3}H already released from total specific ^{3}H. Total specific ^{3}H is defined as the sum of ^{3}H-labeled hormone released during the en-

tire experiment and ^{3}H-labeled hormone remaining in the pituitary explant at the end of the experiment (23).

Results. *Effect of verapamil on [^{3}H]GH and [^{3}H]PRL release in basal rat pituitary perifusion.* In four series of experiments, rat pituitary explants in basal perifusion were exposed to 45-min pulses of 5, 50, or 250 μM verapamil (Fig. 1). Almost no effect of 5 μM verapamil on basal [^{3}H]GH release was observed, whereas [^{3}H]PRL release was inhibited and did not recover. The effect of 50 μM verapamil upon [^{3}H]PRL release did not differ from that of 5 μM verapamil. In contrast, [^{3}H]GH release was stimulated throughout the brief verapamil pulse, and returned to its preverapamil base after the pulse. At a 250 μM concentration, verapamil produced a triphasic effect upon both [^{3}H]GH and [^{3}H]PRL release: initial stimulation, inhibition below the preverapamil base during continued verapamil, and rebound release after verapamil withdrawal. The [^{3}H]GH rebound rose above the preverapamil basal release rate while the [^{3}H]PRL rebound did not.

During prolonged exposure to 50 μM verapamil, [^{3}H]GH release rates returned to preverapamil base (see Fig. 5). Therefore, subsequent experiments were performed using 50 μM verapamil in order to use this initial verapamil-induced increase in [^{3}H]GH release as an internal control for the verapamil activity in each experiment.

Verapamil prevents the inhibition of basal and dbcAMP-stimulated [^{3}H]GH and [^{3}H]PRL release by increased extracellular Ca^{2+}. Increased extracellular Ca^{2+} (5.4 mM) inhibits the release of [^{3}H]PRL and [^{3}H]GH (20) from basal and continuously dbcAMP-stimulated rat pituitaries in perifusion (Figs. 2 and 3). Verapamil (50 μM) prevents those effects of increased extracellular Ca^{2+} (Figs. 2 and 3). In the basally perifused pituitary, inhibition of hormone release by 5.4 mM Ca^{2+} is followed by a temporary rebound increase in the hormone release rate (Fig. 2) when perifusion medium Ca^{2+} is restored to 1.8 mM (20). However, after equilibration (see below) of the perifused pituitaries in 50 μM verapamil, 5.4 mM Ca^{2+} was without effect (Fig. 2). Continuous stimulation of hormone release by 1 mM dbcAMP is markedly inhibited by 5.4 mM Ca^{2+} (20). This inhibition is totally overcome by 50 μM verapamil after whose

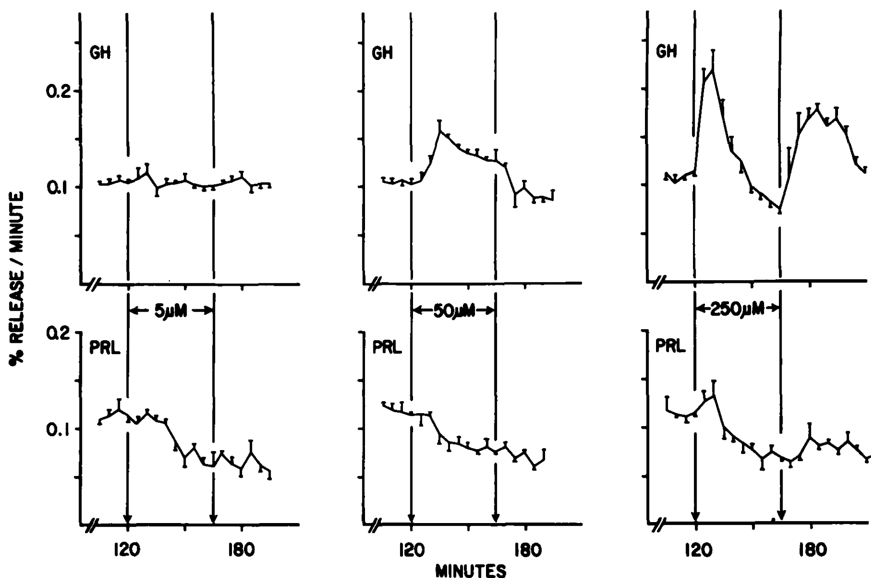

FIG. 1. Effect of 45 min of 5, 50, and 250 μM verapamil on [^{3}H]GH and [^{3}H]PRL release from perifused rat pituitaries (n = 4). In this and in subsequent figures the equilibration period of perifusion is omitted, medium Ca^{2+} is 1.8 mM unless otherwise specified, the downward arrow indicates medium change, GH and PRL are measured independently in the same experiments, and mean ± SE of measurements are presented.

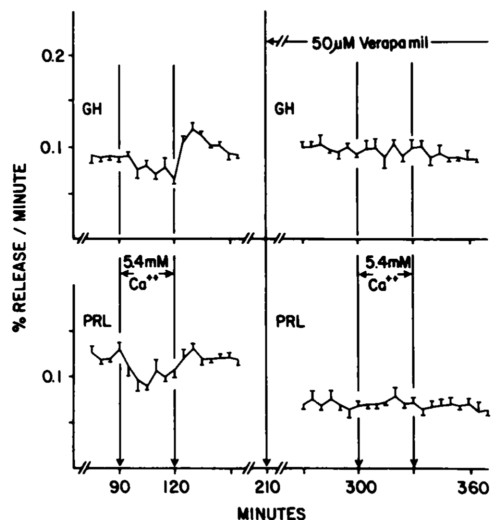

FIG. 2. Effect of 50 μM verapamil on 5.4 mM Ca^{2+}-induced inhibition of [^{3}H]GH and [^{3}H]PRL release from perifused rat pituitaries (n = 3). Both Ca^{2+} pulses are given in the same experiment; data from 155 to 230 min are omitted.

removal [^{3}H]GH release rates immediately return toward the preverapamil, Ca^{2+}-inhibited level (Fig. 3). Verapamil effects on [^{3}H]PRL release are qualitatively similar, but recovery of release rates is only partial.

Verapamil (50 μM) fails to prevent the effect of SRIF on [^{3}H]GH release. SRIF inhibition of [^{3}H]GH release is followed by a rebound release in this system (22). Neither the SRIF-induced inhibition, nor the post-SRIF rebound release, of [^{3}H]GH was prevented by 50 μM verapamil (Fig. 4). SRIF may produce a small, variable decrease of [^{3}H]PRL release in this *in vitro* system (data not shown) but has no significant effect in the presence of 50 μM verapamil (Fig. 4).

Verapamil (50 μM) enhances the stimulatory effect of 1 mM dbcAMP on [^{3}H]GH release by rat pituitaries. [^{3}H]GH release is initially stimulated by 50 μM verapamil (Figs. 1, 5, and 6). With continued perifusion in the presence of verapamil, however, the [^{3}H]GH release rate returns to, and even below, the preverapamil base (0.898 ± 0.021, $P < 0.02$). [^{3}H]PRL release is simply inhibited (0.482 ± 0.073, $P < 0.01$). In separate experiments, the stimulation of such pituitaries by 1 mM dbcAMP, 2 or 4 hr after initiating exposure to 50 μM verapamil, results in [^{3}H]GH release rates with first and second phases [(23) and see Discussion] that regularly exceed those which occur in the absence of exposure to verapamil (Fig. 5). The verapamil-induced enhancement of the [^{3}H]GH response to dbcAMP requires the presence of verapamil. If verapamil is discontinued when dbcAMP is added, the enhanced [^{3}H]GH dbcAMP response does not occur despite 2 hr of verapamil pretreatment (Fig. 5, closed triangles, upper panel). As with basal release, dbcAMP-stimulated release of [^{3}H]PRL from pituitaries exposed to 50 μM verapamil is partially inhibited (Fig. 5).

Verapamil (50 μM) blunts the stimulatory effect of 21 mM K^+ on [^{3}H]GH and [^{3}H]PRL release by perifused rat pituitaries. The brief pulse of hormone release by perifused rat pituitaries stimulated with continuous 21 mM K^+ (26) is inhibited after 2 hr of pretreatment

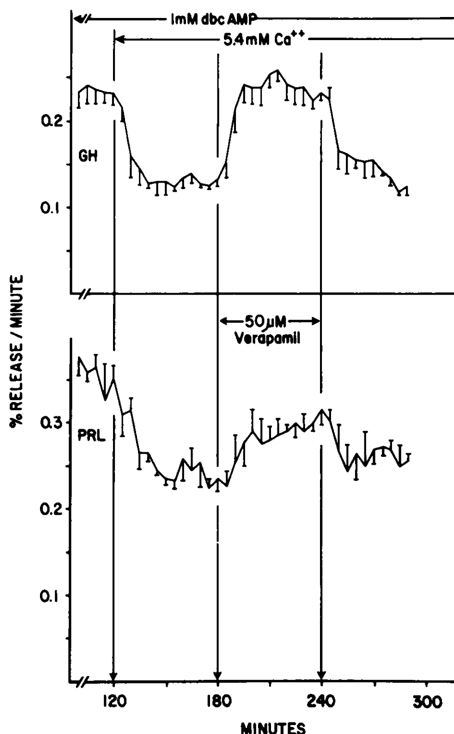

FIG. 3. Effect of 50 μM verapamil on 5.4 mM Ca^{2+}-induced inhibition of [^{3}H]GH and [^{3}H]PRL release from 1 mM dbcAMP-stimulated rat pituitaries in perifusion. There is no difference between mean GH release rates during dbcAMP and verapamil-Ca^{2+}-dbcAMP portions of the protocol.

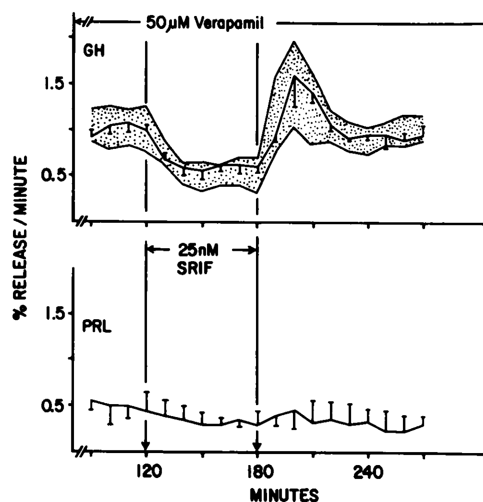

FIG. 4. Effect of a pulse of 25 nM SRIF on [^{3}H]GH and [^{3}H]PRL release from rat pituitaries perifused with continuous 50 μM verapamil (n = 3). The stippled area represents the 95% confidence limits (mean ± 2 SD) of SRIF responses in the absence of verapamil in five previously reported experiments.

with 50 μM verapamil (Fig. 6, right portion of upper and lower panels) in agreement with results obtained by others (18). Inhibition of ^{3}H-labeled hormone release is not observed if the verapamil and K^+ are added simultaneously (Fig. 6, left portion of upper and lower panels). In addition, in contrast to dbcAMP stimulation [(26) and Fig. 5], K^+-induced increases in [^{3}H]GH and [^{3}H]PRL release rates which are not normally sustained (26), are also not enhanced in the presence of 50 μM verapamil (Fig. 6).

Discussion. These data demonstrate that the effects of high concentrations of extracellular Ca^{2+} upon pituitary GH and PRL release *in vitro* can be antagonized by verapamil: (a) prior application of verapamil prevents the effects of increased Ca^{2+} from occurring (Fig. 2), and (b) addition of verapamil to the system already exposed to increased Ca^{2+} rapidly reverses the effects of increased extracellular Ca^{2+} on hormone release (Fig. 3). The Ca^{2+}-induced inhibition is not a nonspecific divalent cation effect (20); $BaCl_2$, for example, stimulates GH release (27) without an associated $^{45}Ca^{2+}$ efflux. In fact, the stimulatory effect of 5.4 mM $BaCl_2$ is not prevented by verapamil (data to be presented elsewhere).

It is possible, then, that the hormone release-inhibitory effects of increased extracellular Ca^{2+} (20) result from an inappropriately high concentration of intracellular Ca^{2+} or an improper distribution of intracellular Ca^{2+} secondary to (a) an increased Ca^{2+} influx, (b) the prevention of adequate Ca^{2+} efflux from the cell, or (c) a combination of the first two possibilities. Since the effect of increased extracellular Ca^{2+} is rapidly reversed by the addition of verapamil, prevention of Ca^{2+} influx appears to be the most likely explanation for verapamil's effects, although an effect of verapamil on intracellular Ca^{2+} distribution cannot be excluded. The rapidity with which normal secretory function is restored (Fig. 3) once increased Ca^{2+} influx is prevented by verapamil (13) also implies the rapid reversibility, perhaps by intracellular redistribution, of the effects of increased intracellular Ca^{2+}, such as the prevention of microtubule-associated intracellular hormone transport (28) and the inhibition of enzymes responsible for intracellular hormone transport processes (29).

The observation that verapamil transiently increased GH release in these basal *in vitro* experiments (Fig. 1, 5, and 6) suggests either a direct effect of verapamil itself or the possibility that the 1.8 mM extracellular Ca^{2+} (Minimum Essential Medium, GIBCO) used *in vitro* is itself supraphysiologic (and therefore slightly inhibitory) to the somatotroph: basal hormone release rates must reflect Ca^{2+} equilibria both across the cell membrane and within intracellular compartments. During continued exposure to verapamil, reequilibration of GH release rates at a level below the preverapamil base (Figs. 5 and 6) could reflect intracellular Ca^{2+} depletion (30) in the face of ongoing verapamil-induced inadequate Ca^{2+} influx. That PRL release is immediately inhibited by verapamil (Figs. 1, 5, and 6) or increased extracellular Ca^{2+} (Fig. 2) reinforces suggestions of intrapituitary heterogeneity of Ca^{2+} dependent mechanisms (11) and their responses to Ca^{2+}-dependent effectors. Verapamil has variable effects across a wide range of tissues (13, 18, 19).

Increased extracellular K^+ and dbcAMP are known to stimulate rapid GH release in this system (23, 26). Continued pituitary exposure to dbcAMP results in a sustained stimulation of GH and PRL release (20, 23). On the

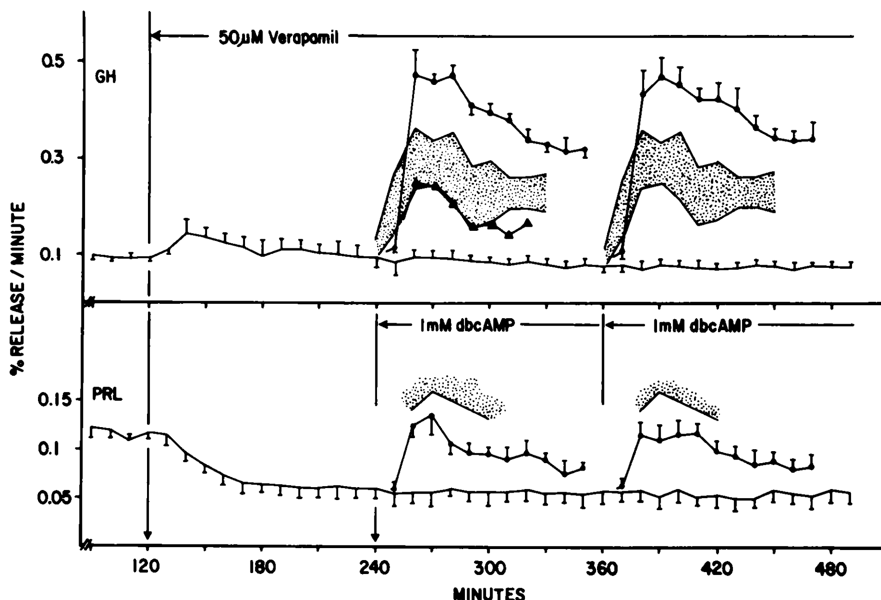

FIG. 5. Effect of prolonged 50 μM verapamil exposure upon [^{3}H]GH and [^{3}H]PRL release from perfused rat pituitaries (unbroken line in upper and lower panels ± SE). In similar but separate experiments, 1 mM dbcAMP was added during verapamil at the fourth or sixth hour (closed circles, $n = 3$). In another set of experiments, verapamil was discontinued at the fourth hour as 1 mM dbcAMP was added (upper panel, closed triangles). The stippled area in the upper panel represents the 95% confidence limits of GH release in response to 1 mM dbcAMP in five separate nonverapamil perifusion experiments. In the lower panel, the 95% confidence limits of the dbcAMP response of PRL are presented, but only the lower portion of those limits are depicted. For GH and PRL these same confidence limits are shown twice for ease of comparison.

other hand, GH and PRL return to pre-K^+ release rates despite continuous exposure to increased extracellular K^+ (26). Further, previous results have suggested that the early release responses produced by these two secretagogues are at least partially independent, originating from overlapping, but distinguishable intracellular sites (26). The present observations (Figs. 5 and 6) that after 2 hr of verapamil pretreatment, the rapid, early phase of dbcAMP-induced GH release was enhanced while rapid K^+-induced GH release was partially inhibited reinforces that suggested distinction.

The separate importance of both transmembrane Ca^{2+} movement and intracellular Ca^{2+} distribution also is suggested in the data presented in Fig. 5. If verapamil's effects result at least in part from preventing Ca^{2+} influx from the extracellular environment and if its (Fig. 6) initial effect upon GH release is stimulatory, a slight inhibitory tone of 1.8 mM extracellular Ca^{2+} is implied. Enhanced GH release in response to 1 mM dbcAMP is then reasonable in such a setting. The early dbcAMP-induced GH response is apparently dependent upon release of Ca^{2+} from intracellular stores (19) and these are apparently not depleted by the presence of verapamil (30). As a result, neither the enhanced dbcAMP-induced release during verapamil (Fig. 5, closed circles) nor the low-normal release immediately after verapamil withdrawal (Fig. 5, closed triangles) is surprising. The development of an inhibition of K^+-induced hormone release during verapamil exposure suggests an alteration in the intracellular state of stored hormone available for non-energy-dependent release by membrane depolarizers (18), this due to altered intracellular Ca^{2+} concentrations and distribution secondary to verapamil (30).

Attempts to measure SRIF effects on $^{45}Ca^{2+}$ flux have been made using only nonphysio-

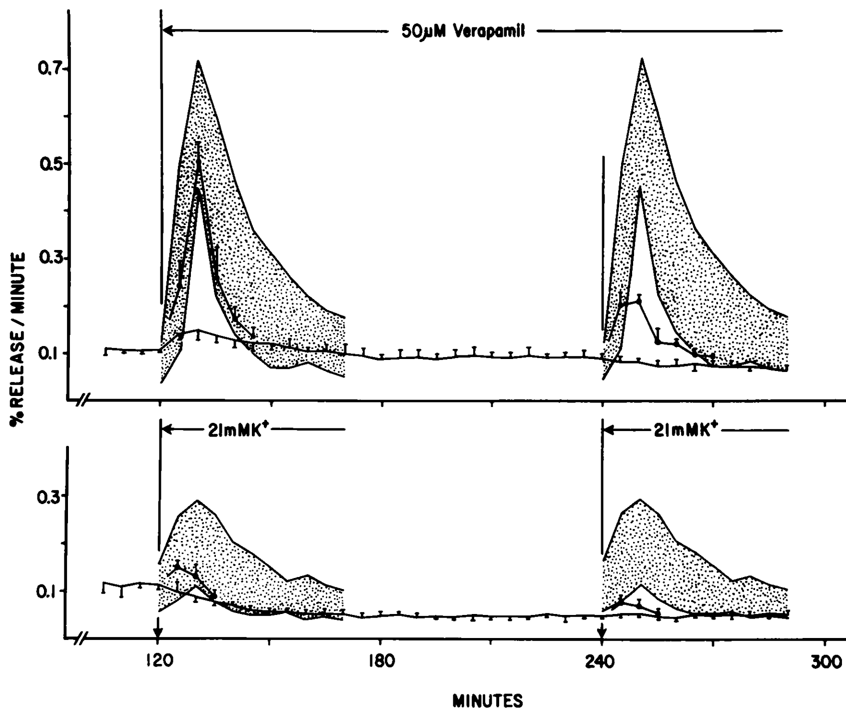

FIG. 6. Effect of 50 μM verapamil on the [^{3}H]GH and [^{3}H]PRL responses to 21 mM K^+ stimulation. The effect of continuous 50 μM verapamil on [^{3}H]GH (upper panel) and [^{3}H]PRL (lower panel) is again presented by the unbroken line (±SE) in each panel. Stimulation of hormone release by 21 mM K^+ in two separate sets of experiments (n = 3, each) at the onset of exposure to verapamil (left portion of both panels) or after 2 hr of exposure to verapamil (right portion of the figure) is depicted (±SE) by the closed circles. The stippled areas in both panels represent the 95% confidence limits of [^{3}H]GH (upper panel) and [^{3}H]PRL (lower panel) release in response to 21 mM K^+ in five separate nonverapamil experiments. For GH and PRL these same confidence limits are shown twice for ease of comparison.

logic (1 μM) SRIF concentrations (19, 21). However, if (micromolar) SRIF indeed reduces K^+-stimulated pituitary (19) and glucose-stimulated pancreatic islet (21, 31) Ca^{2+} incorporation, SRIF effects upon Ca^{2+}-dependent GH release should be mimicked by verapamil. However, in this system, (nanomolar) SRIF inhibits the stimulatory effects of both dbcAMP and depolarizing concentrations of K^+ on GH release (26) (micromolar SRIF behaves no differently in experiments to be presented elsewhere) but verapamil enhances dbcAMP effects on GH release (Fig. 5) while it inhibits K^+ stimulation of GH and PRL release. It may be possible to resolve this apparent discrepancy as follows. Since cytoplasmic Ca^{2+} can derive from extracellular or stored intracellular sources, inhibition of Ca^{2+} incorporation from extracellular sources cannot necessarily be equated with a reduced cytoplasmic Ca^{2+} concentration. Schofield and Bicknell have suggested that SRIF could limit total Ca^{2+} incorporation into the cell either by a direct reduction of cell membrane permeability (similar to verapamil) or by inhibition of Ca^{2+} redistribution from cytoplasm to intracellular stores (19). Although in both cases overall Ca^{2+} uptake would be inhibited, each mechanism could produce different cytoplasmic Ca^{2+} concentrations and, as a result, different release effects. This hypothesis would require SRIF inhibition of GH release despite elevated concentrations of cytoplasmic Ca^{2+}. Evidence for such an effect is available from experiments performed in the presence of high medium Ca^{2+} (20) in which Ca^{2+}- and SRIF-induced inhibition of GH release are additive.

Withdrawal of short-term SRIF (22) or Ca^{2+} (Fig. 2) inhibition of GH release is followed by rebound release. Although 50 μM verapamil prevents the effects of 5.4 mM Ca^{2+} (Fig. 2), it does not prevent SRIF-induced inhibition and rebound release (Fig. 4).

These data are consistent with the possibility that as with SRIF, verapamil's overall effect may result from more than one concentration-dependent action: (a) intracellular events which lead to release may be inhibited by verapamil-mediated prevention of Ca^{2+} uptake, and (b) at the proper concentration, verapamil may inhibit release directly at the membrane where readily releasable hormone may accumulate as it does with SRIF (22, 26).

If high extracellular Ca^{2+} concentrations inhibit hormone release by causing intracellular Ca^{2+} concentrations or distribution which are themselves inhibitory (18, 28, 29, 32), verapamil could reverse these effects. When verapamil administration preceded exposure to increased extracellular Ca^{2+}, verapamil would prevent the rise of intracellular Ca^{2+} (13). When verapamil administration followed exposure to increased extracellular Ca^{2+}, verapamil would permit dissipation of the elevated intracellular Ca^{2+} (30).

In conclusion: (a) presumably by preventing Ca^{2+} entry into the cell, verapamil antagonizes the inhibitory effects of increased (5.4 mM) extracellular Ca^{2+} on basal and stimulated [^{3}H]GH and [^{3}H]PRL release; (b) verapamil itself initially increases [^{3}H]GH release suggesting either a direct verapamil effect or an inhibitory tone secondary to even more physiologic (1.8 mM) medium Ca^{2+} concentrations; (c) verapamil-sensitive Ca^{2+} movement is not necessary for dbcAMP stimulation of GH release; (d) rapid, early GH and PRL release in response to dbcAMP and increased K^+ occur by mechanisms or from sites which exhibit differential Ca^{2+} sensitivity; and (e) SRIF is effective despite the presence of verapamil, suggesting that although each may modify movement and/or distribution of Ca^{2+}, their actions must partially differ.

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