MOLECULAR PHARMACOLOGY, 26:405–413

The Relationship between Phosphatidylinositol Metabolism and Mobilization of Intracellular Calcium Elicited by *Alpha*$_1$-Adrenergic Receptor Stimulation in BC3H-1 Muscle Cells

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Received May 7, 1984; Accepted August 30, 1984

SUMMARY

The BC3H-1 cell is a stable cell line of probable smooth muscle origin which expresses nicotinic acetylcholine, *alpha*$_1$- and *beta*$_2$-adrenergic receptors on its cell surface. Stimulation of the *alpha*$_1$ receptor mobilizes 70% of the intracellular Ca^{2+} within a 2–3-minute interval. To delineate further the linkage between *alpha*$_1$-receptor occupation and response, we have examined the quantitative relationship between fractional occupation of the receptor, the turnover of inositol-containing phospholipids, and the Ca^{2+} efflux. *Alpha*$_1$-receptor activation stimulates the incorporation of [^{3}H]inositol into phosphatidylinositol and the enhanced incorporation is linear over a 60-min interval. In contrast, agonist-elicited increases in hydrolysis of phosphatidylinositol and phosphatidylinositol mono- and bisphosphate develop more slowly, and a 5-min lag in enhanced formation of inositol trisphosphate, inositol bisphosphate, and inositol monophosphate is evident. The increased rate of Ca^{2+} efflux and enhanced rate of inositol incorporation into phosphatidylinositol elicited by phenylephrine exhibit virtually identical dependencies on agonist concentrations. Moreover, fractional inactivation of receptors with phenoxybenzamine shows equivalent increments in the reduction of the two intracellular responses. Both responses are linearly related to the residual receptor sites remaining after fractional inactivation. These findings indicate an absence of a receptor reserve in activating these intracellular events. Moreover, although *alpha* receptor occupation stimulates phosphatidylinositol hydrolysis, no evidence is provided that this event would precede Ca^{2+} release. Should inositol trisphosphate mediate intracellular Ca^{2+} mobilization in these cells, it would be active in extremely low concentrations or occur as a tightly coupled event in a microscopic environment.

INTRODUCTION

Since Hokin and Hokin (1) initially described an enhancement of phosphatidylinositol metabolism upon hormonal stimulation, several hormones and neurotransmitters have been shown to elicit this response (*cf.* refs. 2 and 3). Many of these agents are also believed to elevate the intracellular free calcium concentration in the same tissue. How these two molecular events are causally related within the cell has been an issue of intense debate in recent literature (3–5). Recent findings that inositol trisphosphate in low concentrations will release cellular Ca^{2+} in pancreatic acinar cells and liver suggest, at least in these tissues, that inositol 1,4,5-trisphosphate may be a mediator of intracellular Ca^{2+} release (6–8).

Several investigations have addressed the question of receptor-response coupling in systems expressing *alpha*-adrenergic receptors. Stimulation of *alpha*$_1$-adrenergic receptors in the hepatocyte eventually leads to efflux of intracellular potassium, whereas *alpha*$_1$ activation in the parotid gland leads to secretion in addition to K^+ efflux (9, 10). In these functionally divergent tissues, K^+ efflux and the secretory event are mediated through the release of sequestered Ca^{2+} (9, 10). Vascular smooth muscle also expresses *alpha*$_1$ receptors. Activation of these receptors leads to contraction which is also believed to be a consequence of an increase in free intracellular Ca^{2+} (11, 12). However, little is known about how *alpha*$_1$-agonists affect phosphatidylinositol metabolism in smooth muscle and how this event is related to the mobilization of intracellular Ca^{2+}. A model for studying this relationship can be developed in BC3H-1 cells, which appear to be of

Supported by United States Public Health Service Training Grant GM 07313-09 to S. K. A., an American Heart Association Advanced Postdoctoral Fellowship to R. D. B., and United States Public Health Service Grant HL 25457.

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0026-895X/84/06405-09$02.00/0

smooth muscle origin (13). The BC3H-1 cell line expresses both $alpha_1$- and $beta_2$- adrenergic receptors on the cell surface (14). Agonists of $alpha_1$ receptors activate an increase in the rate of $^{45}Ca^{2+}$ efflux in BC3H-1 cells, which reflects $alpha_1$-receptor mobilization of stored calcium (15). Stimulation of *beta* receptors elevates cellular cAMP concentrations (14). By studying a single cell type in monolayer, the receptor-elicited responses should be a consequence of equivalent exposure of each cell to the agonist. In this study, we employ the BC3H-1 cell line to investigate the linkage between $alpha_1$-adrenergic receptor occupation and the intracellular responses of phosphatidylinositol turnover and calcium mobilization.

MATERIALS AND METHODS

Materials. Phenylephrine·HCl and all lipid standards were obtained from Sigma Chemical Co. (St. Louis, MO). Phentolamine mesylate was a gift from Dr. Paul Insel (Division of Pharmacology, University of California, San Diego). Dowex AG1-X8, 200–400 mesh, formate form, was obtained from Bio-Rad (Richmond, CA). [^{3}H]*myo*-Inositol and [^{3}H]prazosin were obtained from New England Nuclear Co. (Boston, MA). $^{45}Ca^{2+}$ was obtained from Amersham Corporation (Arlington Heights, IL). Details concerning the BC3H-1 cell line, the conditions for growth, and materials used are given in ref. 15. All experiments were performed on day 12–14 confluent cells in 35-mm Petri dishes. All other chemicals used were of reagent grade.

Measurement of [^{3}H]inositol incorporation into BC3H-1 cells. Individual cultures of BC3H-1 cells were transferred from the incubator to a 37° water bath prior to initiating the assay. Within 5 min, and usually within 1 min, the medium was aspirated from the culture dish and the cells rapidly washed with three 2-ml aliquots of buffer (composition in millimolar: NaCl, 140; KCl, 5.4; $CaCl_2$, 1.8; $MgSO_4$, 1.6; Na_2HPO_4, 1.0; D-glucose, 5.5; HEPES,[3] 25.0; bovine serum albumin, 0.06%; pH 7.4). Buffer solution (750 μl) containing [^{3}H] inositol (4 μCi/ml) was immediately placed on the cells and the dish was returned to the water bath for 60 min. At this time, the buffer was replaced with one containing [^{3}H]inositol plus the appropriate ligands; or the culture was rapidly washed with three 2-ml aliquots of buffer before adding 750 μl of buffer containing the appropriate ligands, but no [^{3}H]inositol, as specified. The cultures were further incubated at 37° for specified durations. The assay was terminated by washing the cells rapidly with four 3-ml aliquots of cold buffer, and then the cells were immediately covered with 500 μl of cold methanol. The cells maintained at 0–4° were scraped from the Petri dishes and the dish was washed with an additional 500 μl of cold methanol. A 500-μl aliquot of cold chloroform was added to each sample prior to storage at −20°. All buffer changes and washes took 5–10 sec to complete.

Cells were extracted by published methods (16, 17) with minor modifications. Briefly, the samples were sonicated in chloroform/methanol/2.0 M KCl (5:10:4) using an Ultrasonics sonicator (model W-225R) for 10 sec. The samples were mixed after adjusting the final chloroform/methanol/2.0 M KCl ratio to 10:10:9, and then centrifuged at 2000 × *g* for 15 min to separate the chloroform and aqueous phases. One ml of the upper (aqueous) phase was transferred to a scintillation vial and the remaining aqueous layer was aspirated. The lower (chloroform) phase was washed with 1.0 ml of chloroform/methanol/2.0 M KCl (3:48:47). After clarification of the two phases as before, 0.5 ml of the aqueous phase of each sample was added to the previous aqueous aliquot collected. The remaining aqueous phase was aspirated and the chloroform phase was transferred to a scintillation vial and evaporated to dryness. All extractions were carried out at 0–4°. [^{3}H]Inositol was quantitated in each phase by scintillation counting after correction for counting efficiency. Migration on TLC showed that greater than 95% of the labeled inositol found in the chloroform-containing phase existed as phosphoinositides. Migration positions were established from standard phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate.

Assay of inositol phosphate accumulation. BC3H-1 cultures were loaded with tracer [^{3}H]inositol in growth medium (2 μCi/ml) for 21 hr in the incubator. The cells were placed in fresh medium without tracer 6 hr prior to the assay. Under these conditions, the activity of the phosphoinositides was approximately 60,000 dpm/dish. Each dish contained 0.5 mg of protein. The assay was initiated by washing the cells with three 2-ml aliquots of buffer, and then applying 750 μl of buffer containing 10 mM LiCl plus the appropriate ligands. The dishes were incubated in a 37° water bath for specified lengths of time. Termination of the incubation and cell extraction were the same as outlined above, except for substitution of deionized water for 2.0 M KCl. The aqueous fraction was placed on a 1-ml Dowex column in order to separate the inositol phosphates from free inositol (18). Free inositol was eluted from the column with deionized water. Total inositol phosphates were eluted from the column with 0.1 M formic acid plus 1.0 M ammonium formate. In some experiments inositol monophosphate, inositol bisphosphate, and inositol trisphosphate were fractionated by successively washing the column with 0.1 M formic acid containing 0.2 M ammonium formate, 0.4 M ammonium formate, or 1.0 M ammonium formate, respectively (18). Data are expressed as the ratio of cpm per sample with respect to the number of cpm in control samples with no incubation interval.

$^{45}Ca^{2+}$ efflux measurements. $^{45}Ca^{2+}$ efflux measurements were based on a modification of the methods used in ref. 15. Briefly, the cells were loaded overnight with $^{45}Ca^{2+}$ (5 μCi/ml) in growth medium. After washing the cells to initiate the assay, the cells were incubated with buffer for 10 min in a 37° water bath. This initial incubation in the absence of medium containing $^{45}Ca^{2+}$ removes the rapidly exchanging $^{45}Ca^{2+}$ such that the remaining basal $^{45}Ca^{2+}$ efflux approximates monophasic kinetics. The buffer was replaced with 2 ml of buffer containing appropriate ligands and incubated for 2 min at 37°. The assay was terminated by rapidly washing with buffer containing 5 mM $LaCl_3$. The amount of $^{45}Ca^{2+}$ remaining in the cells was determined by liquid scintillation counting.

Fractional inactivation of $Alpha_1$ receptors by phenoxybenzamine. Phenoxybenzamine stock solutions were prepared in double-distilled water on the day of the experiment and kept on ice. Within 2 min of application to the cells, stock solutions were diluted into buffer without bovine serum albumin at room temperature. Sister cultures were used to examine the effect of $alpha_1$ receptor inactivation by phenoxybenzamine on [^{3}H]prazosin binding on the intact cell, phenylephrine-stimulated [^{3}H]inositol incorporation, and phenylephrine-stimulated $^{45}Ca^{2+}$ efflux. To examine [^{3}H]prazosin binding, cultures were exposed to appropriate concentrations of phenoxybenzamine for specified times. After washing with three 2-ml buffer aliquots, the cultures were incubated with 0.29 nM [^{3}H]prazosin for 60 min at 37°. Free radioligand was removed by rapid 3-ml buffer washes. Nonspecific binding was determined from parallel incubations in 10 μM phentolamine. Data are expressed as B/B_{max}, where B is the amount of [^{3}H]prazosin specifically bound under the experimental conditions and B_{max} is the amount of specific [^{3}H]prazosin binding in the absence of any phenoxybenzamine.

To measure phosphatidylinositol synthesis, cultures that had been incubated with buffer containing [^{3}H]inositol for 60 min at 37° were exposed to phenoxybenzamine for 2 min. The cells were then rapidly washed and a fresh buffer solution containing 6 μM phenylephrine was added. The cultures were incubated for an additional 60 min at 37° before extraction as described above.

To measure phenoxybenzamine inhibition of agonist-elicited $^{45}Ca^{2+}$ efflux, cultures were equilibrated with tracer $^{45}Ca^{2+}$ for at least 16 hr. Cells were then exposed to phenoxybenzamine in buffer containing $^{45}Ca^{2+}$ for 2 min at 37°. After rapid washing, 2 ml of buffer were added to the dishes and the cells were incubated at 37° for an additional 10 min. The buffer was then replaced with 2 ml of buffer containing 6 μM

[3] The abbreviation used is: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

phenylephrine and the cultures were incubated another 2 min at 37°. Termination of this incubation was the same as outlined above.

Calculation of fractional responses. Incorporation of [^{3}H]inositol into phosphatidylinositol lipids (f_E) is expressed as the ratio of the dpm after 60 min of incubation with agonist relative to the dpm of a corresponding culture in the absence of an incubation interval. The basal [^{3}H]inositol incorporation was determined from a sample incubated in an identical fashion in the absence of ligand (f_E^0). Fractional response is expressed as

$$(f_E - f_E^0)/(f_E^{max} - f_E^0) \quad (1)$$

where f_E^{max} is the maximal response.

The rate constant of $^{45}Ca^{2+}$ efflux (k_E) is expressed as the natural log of the ratio of $^{45}Ca^{2+}$ retained following a 2-min agonist exposure relative to controls terminated without a 2-min efflux interval. Basal $^{45}Ca^{2+}$ efflux rate constants (k_E^0) were determined over the same interval with no exposure to ligands. Fractional responses are expressed as

$$(k_E - k_E^0)/(k_E^{max} - k_E^0) \quad (2)$$

RESULTS

Phosphatidylinositol metabolism in BC3H-1 cells. The kinetics of incorporation of [^{3}H]inositol into BC3H-1 cells is illustrated in Fig. 1. The accumulation of [^{3}H]inositol in the aqueous-extractable compartment of the cell is linear for at least a 2-hr period (Fig. 1B). Exposure to the *alpha*-adrenergic agonist phenylephrine does not affect the rate of uptake. Removal of [^{3}H]inositol from the external media of the culture abolishes the uptake and results in a slight decrease in the amount of radioactivity associated with the aqueous-soluble compartment of the cell (Fig. 1D). This loss of radioactivity is negligible over the following 60 min of the assay.

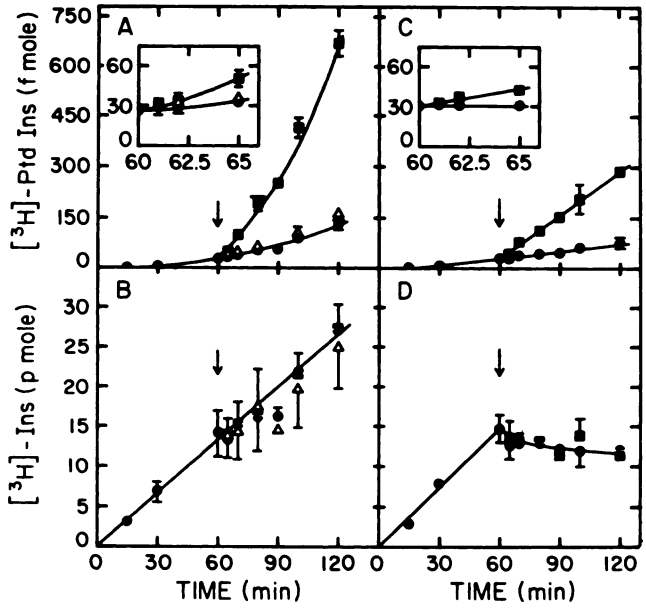

FIG. 1. *Disposition of [^{3}H]inositol after addition of tracer quantities to BC3H-1 cells*

Panels A and C show incorporation into the chloroform-extractable fraction and radioactivity exists as phosphatidylinositol (*Ptd Ins*) lipids. Panels B and D show [^{3}H]inositol remaining in the aqueous phase after extraction. Radioactivity is present as inositol (*Ins*) or inositol polyphosphates. ■, incorporation after addition of 10 μM phenylephrine at the arrow; △, incorporation after addition of 10 μM phenylephrine and 10 μM phentolamine at the arrow; ●, incorporation before and after addition of buffer at the arrow. Panels A and B show incorporation where [^{3}H]inositol remained in the buffer during the entire incubation period. Each point is the mean ± standard error of duplicate culture dishes of eight experiments. Panels C and D show incorporation where [^{3}H]inositol was removed by washing (three times with 2 ml) prior to addition of the compounds at the arrow. Each point is the mean value ± standard error determined from triplicate culture dishes in two separate experiments.

Uptake of [^{3}H]inositol into a cellular aqueous compartment precedes incorporation of [^{3}H]inositol into the chloroform-soluble fraction. Thin layer chromatography on silica gel 60 plates shows that greater than 95% of the radioactivity extracted by chloroform is associated with the phosphoinositides (data not shown). The kinetics of incorporation of [^{3}H]inositol into phosphatidylinositol show an acceleration over this time period presumably because of increasing specific activity of the precursor pool (Fig. 1A). Phenylephrine at 10 μM stimulates a large increase in the rate of [^{3}H]inositol incorporation into phosphatidylinositol. The agonist-stimulated response is completely inhibited by 10 μM phentolamine. There is no perceptible lag in the onset of agonist-stimulated [^{3}H]inositol incorporation, nor does the response abate within the 60-min exposure to phenylephrine. Removal of [^{3}H]inositol from the external media results in a linear incorporation of [^{3}H]inositol into phospholipids, in both the basal incorporation and in the agonist-stimulated incorporation (Fig. 1C). This is likely due to the specific activity of the inositol precursor pool remaining relatively constant over this interval. The rate of [^{3}H]inositol incorporation into phosphatidylinositol should reflect the rate of phosphatidylinositol synthesis from phosphatidic acid and free inositol.

Data in Fig. 2 show that the rate of phosphatidylinositol synthesis is linear with time over a wide range of phenylephrine concentrations. Additionally, there is no perceptible lag in the onset of the augmented [^{3}H]inositol incorporation, even at low phenylephrine concentrations,

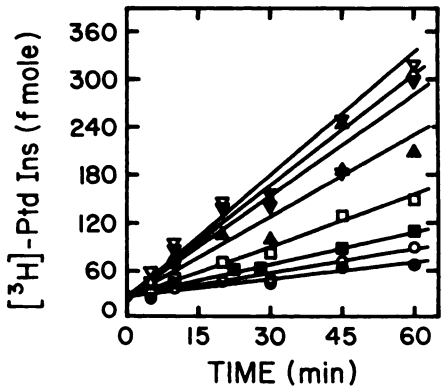

FIG. 2. *Incorporation of [^{3}H]inositol into the chloroform-extractable fraction as a function of phenylephrine concentration*

Samples were incubated with [^{3}H]inositol for 1 hr prior to the addition of phenylephrine. The measurement of inositol incorporation is identical to Fig. 1C. ●, no phenylephrine; ○, 0.03 μM phenylephrine; ■, 0.1 μM phenylephrine; □, 0.3 μM phenylephrine; ▲, 1.0 μM phenylephrine; △, 3.0 μM phenylephrine; ▼, 10 μM phenylephrine; and ▽, 30 μM phenylephrine.

as the data can be extrapolated directly to zero in all cases we have examined.

The above measurements of phosphatidylinositol synthesis may not be expected to reflect precisely the breakdown of inositol phospholipids. It has been hypothesized that the breakdown of phosphatidylinositol 4,5-bisphosphate is a more proximal event to receptor activation than is phosphatidylinositol synthesis (19–21). Synthesis, in turn, may be a compensatory reaction. Phosphatidylinositol 4,5-bisphosphate is broken down by phospholipase C, yielding 1,2-diacylglycerol and inositol 1,4,5-trisphosphate (3, 20, 22). Inositol 1,4,5-trisphosphate is converted rather rapidly to inositol 1-phosphate, the dephosphorylation of which is blocked by LiCl (23, 24). Thus, an accumulation of inositol 1-phosphate is indicative of hydrolysis of all phosphoinositides. Inositol phosphates were separated from inositol on Dowex columns (18) (Fig. 3). Basal accumulation of the combined inositol phosphates in the presence of 10 μM LiCl is low, but linear with time. Stimulation of the cultures with 10 μM phenylephrine increases the rate of accumulation of the combined inositol phosphates, but there is a 5-min lag before the enhanced accumulation can be detected. The magnitude of the enhanced accumulation of inositol phosphates after 30 min is not as great as the magnitude of increased [^{3}H]inositol incorporation into the lipids over a 30-min period of agonist exposure (Fig. 1C). In separate experiments, BC3H-1 cultures were treated as above, except one-half of the sister culture dishes were exposed to buffer containing LiCl for 4 min prior to agonist addition (Fig. 4A and B). The results indicate that prior exposure of the cells to LiCl has no effect on the overall inositol phosphate accumulation elicited by agonists.

The three inositol phosphates, inositol monophosphate, inositol bisphosphate, and inositol trisphosphate, were also fractionated and analyzed to ensure that agonist-induced changes in either of the two inositol polyphosphates were not overshadowed by the large abundance of inositol monophosphate (Fig. 4B). Again, no significant increases in any of the individual inositol phosphates could be detected at 5 min, yet at 30 min the agonists enhanced the accumulation of all three inositol phosphates. In separate experiments, cells were extracted with trichloroacetic acid instead of chloroform/methanol/water. Although this approximately doubled the radioactivity recovered in each inositol phosphate peak, significant changes in inositol phosphates were again not observed prior to 5 min of agonist exposure (not shown).

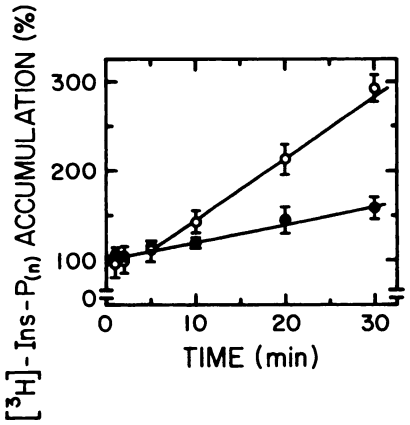

FIG. 3. *Incorporation of [^{3}H]inositol into inositol phosphates*
Samples were incubated with [^{3}H]inositol in medium for 21 hr and placed in fresh medium for 6 hr prior to initiation of the experiment. Inositol phosphates were measured as described in methods. ●, addition of 10 μM phenylephrine; ○, addition of buffer only. Data are expressed as a percentage of the initial value of [^{3}H]inositol phosphate and shown as means ± standard error for duplicate culture dishes in four experiments.

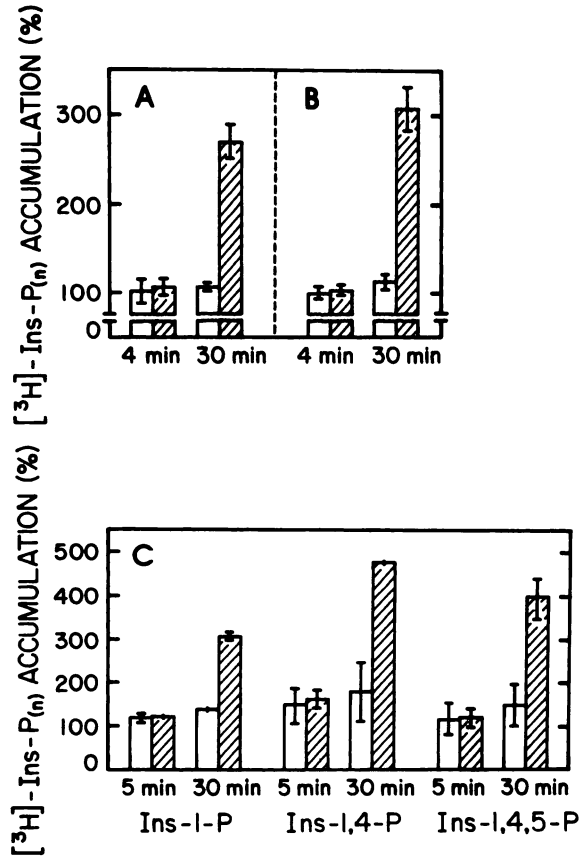

FIG. 4. *Incorporation of [^{3}H]inositol into inositol phosphates*
A and B: accumulation of total inositol phosphates. The bar graphs in Panel A show the inositol phosphate incorporation when 10 mM Li^+ was added at time zero. The bar graphs in Panel B show incorporation when 10 mM Li^+ was added 4 min prior to agonist addition. Clear symbols, no agonist addition; hatched symbols, 10 μM phenylephrine. Data are expressed as a percentage of the initial value of [^{3}H]inositol phosphate. C: accumulation of individual inositol phosphates. Conditions and symbols are identical to those shown in A. For inositol monophosphate, inositol bisphosphate, and inositol trisphosphate, the 100% value represents 300, 25, and 12 cpm/dish above blank counts, respectively. Each mean value ± standard error for the three panels was determined from duplicate culture dishes done in three separate experiments.

Phenylephrine-stimulated efflux of $^{45}Ca^{2+}$. Phenylephrine-elicited $^{45}Ca^{2+}$ efflux from BC3H-1 cells was monitored following an initial incubation of the cells in buffer without agonist for 10 min (see Materials and Methods). The initial 10-min interval eliminates a component of rapidly exchanging Ca^{2+} (cf. ref. 15) which interferes with the estimation of the agonist-elicited response. With

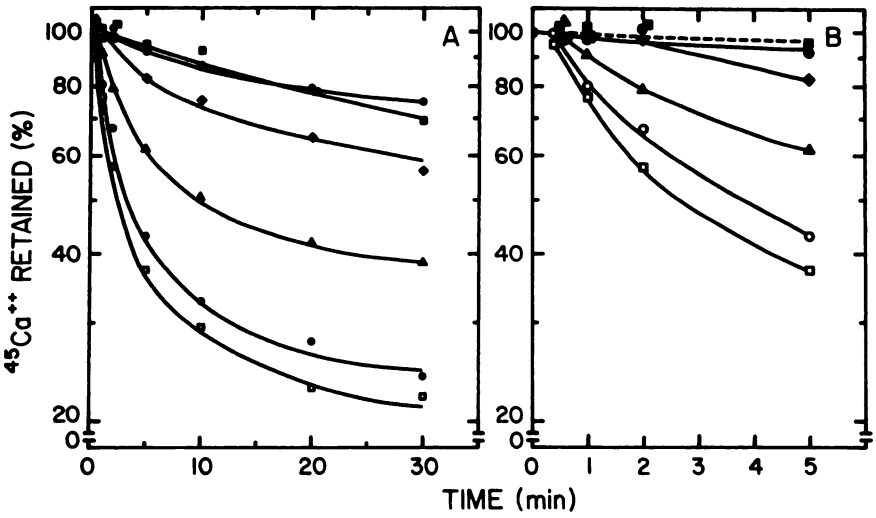

FIG. 5. *Efflux of $^{45}Ca^{2+}$ in response to activation by phenylephrine*

BC3H-1 cells were incubated with tracer $^{45}Ca^{2+}$ for 16 hr to achieve isotopic equilibrium. $^{45}Ca^{2+}$ in the medium was removed in three washes of buffer. A fourth wash of buffer was maintained on the cells at 37° for 10 min before replacement with an identical buffer containing phenylephrine. Efflux was measured over the specified interval and terminated with a $LaCl_3$ wash. Panel B shows an expansion of data over short time intervals. ●, no phenylephrine; ■, 0.1 μM phenylephrine; ◆, 0.3 μM phenylephrine; ▲, 1.0 μM phenylephrine; ○, 3.0 μM phenylephrine; □, 10 μM phenylephrine.

the above protocol efflux can be measured at intervals of 1 min and a short lag (30 sec) in response is apparent (Fig. 5). Nevertheless, agonist-elicited efflux is rapid and appears essentially complete in 10 min. Initial rates were calculated from retained Ca^{2+} 2 min after addition of agonist.

Phenylephrine concentration dependence of $alpha_1$-receptor mediated responses. The concentration-dependences of phenylephrine on the *$alpha_1$* receptor-mediated events, phosphatidylinositol synthesis and $^{45}Ca^{2+}$ efflux, in the BC3H-1 system are shown in Fig. 6A and B. The K_{act} (concentration of half-maximal stimulation) for phenylephrine-stimulated phosphatidylinositol synthesis is 0.83 μM and the Hill coefficient (determined as in ref. 25) is 0.85 ± 0.02. The K_{act} for phenylephrine stimulation of $^{45}Ca^{2+}$ efflux is 0.90 μM and the Hill coefficient is 1.04 ± 0.04. Plotting the respective responses against one another (Fig. 6C) yields a linear relationship between fractional activation of [^{3}H]inositol incorporation into the phospholipids and fractional activation of $^{45}Ca^{2+}$ efflux. Linear regression analysis produces a slope of 0.96 with a correlation coefficient of 0.993. Thus, the concentration dependencies appear essentially identical. To further investigate the relationship between receptor number and the individual postreceptor events, the fractional receptor occupation was correlated with the fractional responses as described below.

Fractional inactivation of $alpha_1$-adrenergic receptors. Phenoxybenzamine has been used as an alkylating agent to irreversibly inactivate fractions of the *$alpha_1$* receptor population (26). This compound has been utilized extensively in intact tissues (26–28), but the relative rates of reversible and irreversible binding of phenoxybenzamine have not been analyzed in monolayers of cells where each cell would receive equivalent exposure to the alkylating agent. To investigate this, BC3H-1 cells were incubated with specified phenoxybenzamine concentrations over short intervals. The unalkylated sites were determined by [^{3}H]prazosin binding after repeated washing of the cells (Fig. 7).

Irreversible inactivation of receptors (R) by phenoxybenzamine (Pbz) should proceed through a reversible complex ($Pbz\cdots R$) to form the alkylated product (Pbz-R) as follows:

$$Pbz + R \overset{K_R}{\rightleftharpoons} Pbz\cdots R \overset{k_3}{\rightarrow} Pbz - R \qquad (3)$$

Since the rate of irreversible inactivation does not appear to become limiting at phenoxybenzamine concentrations up to 1.0 μM, it is apparent that the reversible dissociation constant (K_R) for phenoxybenzamine binding should equal or exceed this value. Furthermore, the limiting rate constant for irreversible alkylation (k_3) is greater than 0.02 sec^{-1} and cannot be estimated from manual addition of phenoxybenzamine to the cells. Thus, under our conditions of measurement, the rate of receptor inactivation will depend on both the reversible and irreversible steps (29). If the rate were independent of phenoxybenzamine concentration, inactivation would be solely dependent on k_3.

Following a 2-min exposure to varying concentrations of phenoxybenzamine and rapid washing, the extent of receptor inactivation was determined by [^{3}H]prazosin binding to the remaining receptor sites (Fig. 8A). The phenoxybenzamine concentration necessary to block half of the [^{3}H]prazosin sites in 2 min is 12.1 nM. The effect of partial *$alpha_1$* receptor inactivation on phenylephrine-stimulated [^{3}H]inositol incorporation into phosphatidylinositol and $^{45}Ca^{2+}$ efflux are shown in Fig. 8B and C. The IC_{50} for [^{3}H]inositol incorporation is 12.8 nM phenoxybenzamine and the IC_{50} for $^{45}Ca^{2+}$ efflux is 12.5 nM phenoxybenzamine. Correlation of fractional inhibition of each response to fractional inhibition of [^{3}H] prazosin binding approximated a unitary relationship (Fig. 9A and B) in each case. The correlation of inhibition

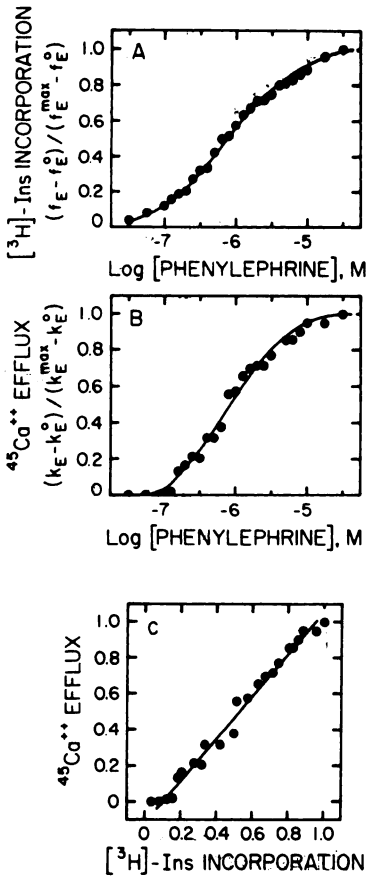

FIG. 6. *Phenylephrine concentration dependence for [^{3}H]inositol incorporation into phosphatidylinositol and the release of intracellular Ca^{2+} in BC3H-1 cells*

A: [^{3}H]inositol incorporation into phosphatidylinositol. Conditions for measurement are identical to Figs. 1C and 2. Measurements were made 60 min after agonist addition, f_E is the ratio of agonist-stimulated incorporation at 60 min relative to time zero. f_E^0 is the ratio of incorporation in the absence of agonist. f_E^{max} is the ratio of incorporation at agonist concentrations yielding a maximum response (100 μM phenylephrine). B: efflux of tracer $^{45}Ca^{2+}$ in response to phenylephrine. k_E is the rate constant for efflux at the specified agonist concentration, k_E^{max} is the rate at maximum agonist concentration, and k_E^0 is the value in the absence of agonist. For A and B, each point comes from at least two experiments using duplicate culture dishes. C: correlation between the fractional responses. Regression analysis yields a slope of 0.96 and a regression coefficient of 0.993.

of [^{3}H]inositol incorporation and [^{3}H]prazosin binding has a slope of 1.16, an abscissa intercept of 0.06, and a correlation coefficient of 0.974. The slope of the line formed by a corresponding analysis of inhibition of $^{45}Ca^{2+}$ efflux is 1.24, and the correlation coefficient is 0.970. The x intercept in this case is 0.11. Comparison of the fractional inhibition of [^{3}H]inositol incorporation to fractional inhibition of $^{45}Ca^{2+}$ efflux (Fig. 9C) yields a slope of 1.04 and a correlation coefficient of 0.985. Thus, a close correlation between receptor occupancy and the functional responses of Ca^{2+} efflux and phosphatidylinositol synthesis can be demonstrated in BC3H-1 cells.

DISCUSSION

The possibility that receptor-mediated phosphatidylinositol metabolism is involved in the coupling of recep-

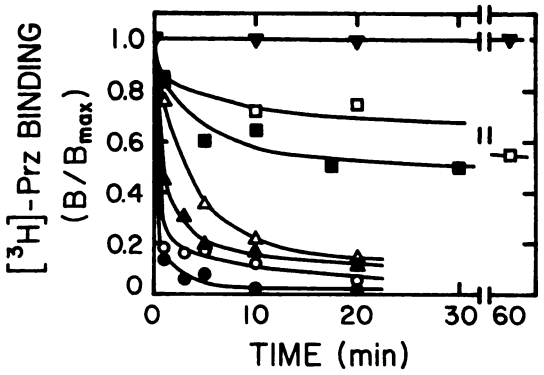

FIG. 7. *The rate of phenoxybenzamine inhibition of prazosin binding*

Phenoxybenzamine at the designated concentrations was incubated with BC3H-1 cells for the specified times and washed from the culture dishes with three 2-ml volumes. [^{3}H]Prazosin (*Prz*) binding was measured after a 60-min incubation as detailed in Materials and Methods. ▼, incubation with buffer alone; □, 0.3 nM phenoxybenzamine; ■, 3.0 nM phenoxybenzamine; △, 10 nM phenoxybenzamine; ▲, 30 nM phenoxybenzamine; ○, 100 nM phenoxybenzamine; ●, 1.0 μM phenoxybenzamine.

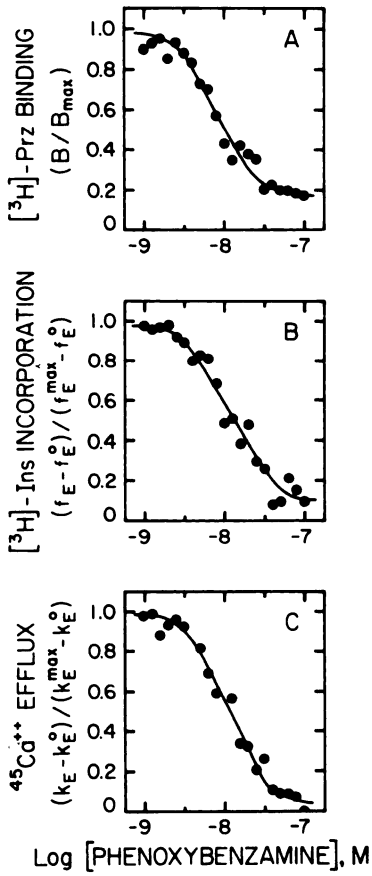

FIG. 8. *The influence of prior exposure of BC3H-1 cells to phenoxybenzamine on the residual binding sites and functional responses*

A:[^{3}H]prazosin binding; B: [^{3}H]inositol incorporation into phosphatidylinositol; C: $^{45}Ca^{2+}$ efflux. Cells were incubated with the specified concentration of phenoxybenzamine for 2 min and washed three times with 2 ml of buffer, and the corresponding assay was carried out as specified in Materials and Methods. Data are averages of two experiments consisting of duplicate culture dishes.

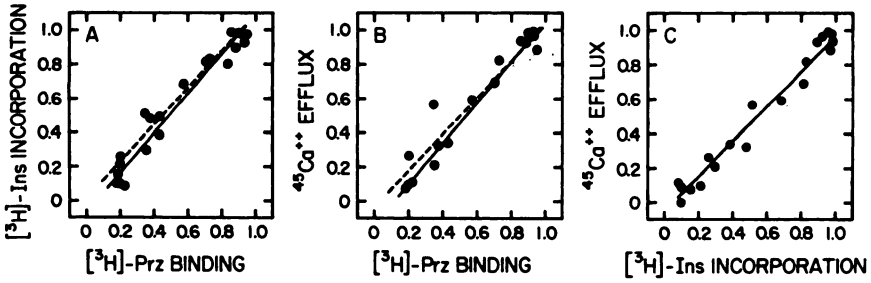

FIG. 9. *Correlations between prazosin occupation of alpha$_1$ receptor sites, [^{3}H]inositol incorporation, and $^{45}Ca^{2+}$ efflux after the fractional inactivation of receptor sites by phenoxybenzamine*

The solid lines are taken from regression analysis of the data in fig. 8. The dotted lines constitute a hypothetical regression analysis assuming that 15% of the prazosin binding sites are unavailable to phenoxybenzamine (see text for explanation).

tor activation to Ca^{2+} mobilization has received strong support from recent studies in saponin-treated cells in which their membranes are rendered permeable to inositol trisphosphate (6–8). In pancreatic acinar cells and hepatocytes, substantial release of intracellular Ca^{2+} can be achieved at concentrations as low as 0.1 μM inositol trisphosphate. Moreover, upon stimulation of cell surface receptors, a rapid accumulation of inositol phosphates has been detected (20, 23, 24). Thus, it has been proposed (20) that inositol 1,4,5-trisphosphate serves as an intracellular mediator of Ca^{2+} release. Whether inositol 1,4,5-trisphosphate is the predominant mediator of the release of Ca^{2+} in smooth muscle has not been investigated in detail. Adenine nucleotides, for example, are effective in releasing Ca^{2+} from T-tubules isolated from skeletal muscle (30). A recent report has demonstrated the release of Ca^{2+} from skinned coronary artery cells by application of inositol 1,4,5-trisphosphate (31).

The BC3H-1 cell line provides a model system for investigating the relationships between *alpha$_1$* receptor activation, phosphatidylinositol metabolism, and calcium mobilization in a smooth muscle-type system. Large quantities of intracellular calcium are mobilized upon *alpha* receptor stimulation (15), and each cell in the monolayer should receive an identical exposure to agonist.

Upon investigating the *alpha$_1$* receptor activation of phosphatidylinositol metabolism in BC3H-1 cultures, we observed a disparity between the kinetics of phenylephrine-stimulated phosphatidylinositol synthesis and the stimulated breakdown. No delay in the onset of *alpha* agonist-stimulated phosphatidylinositol synthesis was evident, yet we found an apparent 5-min delay between agonist addition and initiation of the accumulation of the inositol phosphates. It would seem that these results cannot be explained on the basis of inadequate labeling of precursor pools since the cells were incubated with [^{3}H]inositol for 21 hr. While isotopic equilibrium is not achieved in this interval, substantial fractional labeling of precursor pools should be achieved. In addition, labeling with 10 times the concentration of labeled inositol did not alter the lag in development of agonist-stimulated inositol phosphate concentrations. Our results were rather surprising, as data available from hepatocytes (19, 21) and other systems (20, 31–33) suggest that the production of inositol 1,4,5-trisphosphate is a rapid and presumably proximal event of receptor activation, and occurs prior to the resynthesis of phosphatidylinositol. It is possible that a large amount of inositol trisphosphate need not be generated in smooth muscle, as a small, but highly localized, production of this mediator could lead to calcium mobilization from nearby stores. Smooth muscle cells possess specialized invaginations of the plasma membranes called caveoli which appear in close proximity to the underlying sarcoplasmic reticulum and stores of calcium (34). If *alpha$_1$* receptors were localized near caveoli, receptor stimulation could lead to the production of small amounts of inositol trisphosphate near the sarcoplasmic reticulum, mobilizing calcium from these stores and releasing it directly at the site of its action. In nonmuscle cells, the extent of specialization of Ca^{2+} storage beneath the cell membrane may differ and a more diffuse production of inositol trisphosphate may be necessary to release an equivalent Ca^{2+} concentration throughout the cell. In any event, considerable signal amplification would have to take place in the coupling between inositol trisphosphate generation and calcium mobilization, rather than solely relying on inositol trisphosphate generation itself for amplification.

A second and possibly related explanation for the slow accumulation of the inositol phosphates would invoke the requirement of an intervening mediator. For example, *alpha$_1$*-adrenergic stimulation could elicit the release of arachidonic acid metabolites which in turn stimulate the breakdown of the polyphosphoinositides. Thromboxane-stimulated phosphatidylinositol breakdown has been demonstrated in platelets (35).

A third explanation for the observed lag in agonist-stimulated inositol phosphate production may be that the isomeric forms of inositol phosphate produced at early times do not lead to the accumulation of inositol 1-phosphates. Should the inositol polyphosphates not contain the 1-phosphate moiety, dephosphorylation of these isomers would not be sensitive to LiCl inhibition and hence they would be susceptible to rapid breakdown. Evidence has been put forth for agonist-activated phospholipase D activity in neutrophils upon the addition of chemoattractants (36). This enzyme would cause the production of inositol phosphates lacking phosphate substitution at the 1-position on the inositol ring. High phospholipase D activity in BC3H-1 cells is possible as there is a larger relative accumulation of inositol bis-

phosphate than inositol mono- and trisphosphates (Fig. 4). However, the isomeric forms of these compounds have not been separated and analyzed.

Since calcium has been shown to inhibit synthesis of phosphatidylinositol from CDP-diacylglycerol and inositol, it has been suggested that measuring the amount of [^{3}H]inositol label associated with the phospholipids may provide misleading estimates of hormone responses (3, 37, 38). However, our studies clearly show an increase of [^{3}H]inositol incorporation into phosphatidylinositol concomitant with the increased calcium mobilization. In addition, the studies of Egawa *et al.* (37) indicate that the magnitude of the change in specific activity of [^{3}H] phosphatidylinositol stimulated by agonist is the same in the absence or the presence of calcium. Furthermore, basal breakdown of radiolabeled phosphatidylinositol should be negligible during short periods of [^{3}H]inositol labeling (Figs. 1 and 2). Longer periods of incubation with [^{3}H]inositol should label a substantial fraction of the phosphatidylinositol lipid pool, allowing accurate measurement of phosphoinositide breakdown by monitoring the accumulation of inositol phosphates (Figs. 3 and 4). Thus, by selection of proper conditions, enhanced incorporation of [^{3}H]inositol into phosphatidylinositol should accurately reflect *alpha*$_1$ agonist-activated phosphatidylinositol synthesis in BC3H-1 cells.

We have observed a unitary relationship between *alpha*$_1$-adrenergic activation of phosphatidylinositol synthesis and calcium mobilization in the BC3H-1 system, as determined from the phenylephrine concentration dependence of fractional activation of the two responses (Fig. 6) and by the fractional inactivation of the responses by phenoxybenzamine (Fig. 9). Furthermore, fractional activation of each response appears to be closely dependent upon fractional occupancy of sites on the *alpha*$_1$ receptor. A slight discrepancy in this latter relationship exists, however, as phenoxybenzamine concentrations which fully inactivate phenylephrine stimulated phosphatidylinositol synthesis and $^{45}Ca^{2+}$ efflux inhibit only ~85% of the [^{3}H]prazosin binding (Fig. 8A and B). It may be that the [^{3}H]prazosin penetrates to intracellular binding sites during the 1-hr incubation period with the cultures. With prior dissolution of phenoxybenzamine, the aziridinium ion should be fully formed prior to its addition to the cells. Therefore, minimal penetration across the cell membrane in a 2-min interval would be expected. If we assume that the remaining 15% of [^{3}H]prazosin-binding sites are not accessible to agonist, the data can be recalculated such that maximal inhibition of [^{3}H]prazosin binding by phenoxybenzamine is the baseline. The resulting modification allows intersection close to the origin for *alpha*$_1$ receptor occupancy with phosphatidylinositol synthesis and $^{45}Ca^{2+}$ efflux (dotted line in Fig. 9). Irrespective of this small discrepancy, the above data correspond closely to a scheme in which the fractional occupation of each site on the receptor gives an equal increment in the two responses.

Should the adrenergic receptor be an oligomeric protein with occupation of multiple sites required for activation and inactivation occurring at a single site, then inactivation by phenoxybenzamine would yield a parabolic relationship between the number of available receptor sites and the measured fractional responses. With random site occupation by phenoxybenzamine, the fractional response (k/k_{max}) would be described by $(1 - y)^n$ where y is the fractional occupation by phenoxybenzamine and n is the number of sites in the oligomer (39). On the other hand, should a receptor reserve exist in the system, a curvilinear function in the opposite sense would be expected. In this case, k/k_{max} would equal $1 - y^m$.[4] Over the years, considerable attention has been accorded to "spare" receptors in systems stimulated by adrenergic agonists (27, 40–42). In the isolated rabbit aorta, phenoxybenzamine inhibition shifts the response to agonists to substantially higher agonist concentrations. This finding, along with the observation that the efficacies of full agonists vary by a factor of 10, is indicative of a receptor reserve (41). Similar behavior to phenoxybenzamine antagonism has been noted in the isolated vas deferens (42). Minneman and colleagues (27, 42) have shown in the vas deferens that nearly complete blockade of the contractile response by phenoxybenzamine can be achieved when a small fraction of total binding sites measured in the homogenate are inactivated.

Receptor inactivation studies on intact tissue encounter diffusional limitations for ligand access to cells removed from the surface of the preparation, which would not be expected to be equivalent for agonists and antagonists. Moreover, activation may propagate from electrotonic coupling between cells so that the initial activation by agonist on each cell is not identical. Working with cells in monolayer eliminates these complications, and the relationship between receptor occupation and the two intracellular events we have measured argues against the number of receptors available to agonist greatly exceeding the required number necessary to elicit a maximal response.

The present data are not sufficient to delineate the functional relationship between agonist-elicited phosphoinositide metabolism and calcium mobilization in BC3H-1 cells. However, we do observe differences in the kinetic behavior of the two responses. The onset of phosphatidylinositol synthesis elicited by *alpha*$_1$ agonists is rapid and the enhanced synthesis continues linearly with time up to at least 60 min, although the cumulative response is small in the first few minutes after agonist application. Agonist-stimulated appearance of the inositol phosphates exhibits a distinct 5-min lag before significant increases can be detected. In contrast, agonist-stimulated $^{45}Ca^{2+}$ efflux exhibits only a brief delay of 30 sec prior to onset, following which both unidirectional efflux and net depletion of releasable calcium are already complete within 5–10 min (15). This behavior is consistent with calcium release occurring from intracellular stores near the locus where it is actively extruded

[4] In classical spare receptor theory (40), m will be greater than 1.0 and depend on the efficacy of the agonist and the ratio of agonist concentration relative to its dissociation constant (G. Amitai, R. D. Brown and P. Taylor, submitted for publication).

from the cells. In fact, the short apparent delay in efflux may well relate to the transport process rather than release of calcium from sequestered stores. Recent studies in other systems using the calcium-sensitive fluorescent dye Quin 2 demonstrate that intracellular free calcium concentrations increase within 2–5 sec following agonist stimulation and return to basal levels within a few minutes (43–45).

At this point, it can be concluded that enhanced increments of agonist-stimulated phosphatidylinositol synthesis and calcium mobilization are linked to equivalent fractions of *alpha*$_1$-adrenergic receptor activation in BC3H-1 muscle cells. The responses may occur in parallel and from existing findings it is not possible to demonstrate an interdependence between them.

ACKNOWLEDGMENTS

We thank Drs. Joan Heller Brown and Susan B. Masters for valuable discussions, and Karen D. Berger for her expert assistance in maintenance of the BC3H-1 cell line.

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