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MOLECULAR PHARMACOLOGY, 29:531-539

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α_1-Adrenergic and H1-Histamine Receptor Control of Intracellular Ca^{2+} in a Muscle Cell Line: The Influence of Prior Agonist Exposure on Receptor Responsiveness

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Received October 15, 1985; Accepted March 21, 1986

SUMMARY

Activation of α_1-adrenergic receptors in BC3H-1 muscle cells has been shown previously to mobilize intracellular Ca^{2+}, which can be monitored as enhanced $^{45}Ca^{2+}$ unidirectional efflux. We report here that histamine also stimulates $^{45}Ca^{2+}$ efflux in these cells (K_{act} = 5.50 μM, n_H = 0.94 ± 0.04), reflecting mobilization of intracellular Ca^{2+} from a source similar to that accessed by α_1-adrenergic receptor activation. In addition, histamine stimulates substantial transmembrane $^{45}Ca^{2+}$ influx into BC3H-1 cells. The actions of histamine are inhibited by the H_1-selective antagonist, diphenhydramine (IC_{50} = 1.01 μM), but are unaffected by the H2-selective antagonist, cimetidine (1 nM–10 μM) indicating that histamine regulates cellular Ca^{2+} via a functional H1 receptor. The presence of independent receptor types which mobilize Ca^{2+} originating from common intracellular stores has been exploited in order to evaluate the determinants of receptor responsiveness following prior agonist exposure. After exposure with $^{45}Ca^{2+}$ to achieve radioisotopic equilibrium, 30 min incubation with increasing concentrations of norepinephrine reduces to similar extents (30–40%) the unidirectional $^{45}Ca^{2+}$ efflux responses to subsequent challenges by maximally effective concentrations of norepinephrine or histamine. The decrement in response following norepinephrine exposure appears to reflect an altered disposition of agonist-sensitive intracellular Ca^{2+}, whereas the concentration dependence for α_1-adrenergic receptor activation remains unchanged. Prior exposure of cells to increasing concentrations of histamine also reduces the efflux response to norepinephrine challenge (~30% decrease), whereas the response to subsequent histamine challenge is specifically and completely abolished. The loss of histamine responsiveness is accompanied by a marked shift in the concentration dependence for histamine receptor activation toward higher histamine concentrations. These results indicate that substantial α_1-receptor responsiveness is maintained following agonist exposure and that the observed reduction in response occurs distally to the receptor itself at some point common to α_1- and H1-receptor-effector coupling. By contrast, the H1-histamine receptor exhibits refractoriness, indicative of agonist-induced receptor desensitization.

The contractile state of vascular smooth muscle depends critically upon the concentration of free intracellular Ca^{2+} (1). Receptor-mediated responses which regulate vascular contraction frequently intersect at the level of intracellular Ca^{2+} availability to the contractile apparatus. The contractile state in vascular tissue thus represents an interplay among multiple drugs or hormones whose receptors couple to common intracellular responses.

An additional important consideration may be that cellular responsiveness to receptor activation depends on the past history of exposure to agonist. Thus, prior agonist exposure could result in desensitization at the level of the receptor itself, as has been observed for the nicotinic acetylcholine receptor (2, 3) and the β-adrenergic receptors (4, 5). Alternatively, agonist exposure could alter the disposition of intracellular Ca^{2+}, reducing functional response by acting distally to the receptor at the endpoint of intracellular Ca^{2+} availability. This question is of particular interest in the interaction of the neurotransmitter, norepinephrine, with postsynaptic α_1-adrenergic receptors, which exert tonic control of vascular resistance.

The BC3H-1 cell line has been used previously as a model for study of the relationship between α_1-adrenergic receptor activation and the mobilization of intracellular Ca^{2+} (6–8). The cells are genetically identical and grow in monolayer, enabling uniform exposure of the receptor population to agonists. The BC3H-1 cell line thus offers an attractive system in which to examine the influence of prior agonist exposure upon receptor responsiveness. We report here the characterization of functional H1-histaminergic receptors in these cells which access an intracellular Ca^{2+} store similar to that mobilized upon α_1-receptor activation. In addition, activation of H1-receptors

This work was supported by Grant-in-Aid 84-S105 from the American Heart Association, California Affiliate, and United States Public Health Service Grant HL-25457.

ABBREVIATIONS: PB, physiological buffer; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylene-bis-(oxyethylenenitrile)] tetraacetic acid.

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promotes transmembrane Ca^{2+} entry into the cell. The presence of independent receptor types sharing a common intracellular Ca^{2+} store has been utilized to investigate the basis of agonist-induced alterations in α_1- and H1-receptor responsiveness. A preliminary report of this work has appeared previously (9).

Experimental Procedures

Materials. Histamine·2HCl, *l*-norepinephrine·*d*-bitartrate, *l*-phenylephrine·HCl, diphenhydamine·HCl, cimetidine, superoxide dismutase, catalase, and $LaCl_3 \cdot 7H_2O$ were purchased from Sigma Chemical Co. $^{45}Ca^{2+}$ was purchased from Amersham. Cell culture media and fetal bovine serum were obtained from Gibco (Grand Island, NY). All other chemicals were reagent grade.

Cell culture. Propagation of the BC3H-1 cell line expressing α_1- and β_2-adrenergic receptors and growth of experimental cultures was performed as described in our previous study (6).

Measurement of undirectional $^{45}Ca^{2+}$ fluxes. Procedures for equilibrating BC3H-1 cultures with tracer $^{45}Ca^{2+}$ and measurement of agonist-stimulated $^{45}Ca^{2+}$ efflux were described previously (6–8). In order to obtain more precise measurements of agonist-stimulated $^{45}Ca^{2+}$ efflux from slowly exchanging intracellular compartments, cultures loaded overnight with $^{45}Ca^{2+}$-supplemented growth medium were subjected to an initial 10-min incubation in PB (composition in mM: NaCl, 140; KCl, 5.4; $CaCl_2$, 1.8; $MgCl_2$, 1.6; D-glucose, 5.5; HEPES, 25, pH 7.4) to remove rapidly exchanging cellular $^{45}Ca^{2+}$ (8). Agonist-stimulated efflux was then initiated by replacing the PB with a fresh 2-ml aliquot of PB containing specified ligand concentrations, and efflux was allowed to proceed for indicated time intervals at 37°.

Measurements of cellular $^{45}Ca^{2+}$ content at radioisotopic equilibrium were also performed by a slight modification of our previous method (6). Cultures were equilibrated overnight as usual with 1.0 ml of growth medium containing tracer $^{45}Ca^{2+}$ buffered by $NaHCO_3$-CO_2 exchange in a controlled atmosphere incubator (88% humidified air/12% CO_2 at 37°). Cultures were then transferred to a 37° water bath under room atmosphere and briefly washed with 1.0 ml of fresh $^{45}Ca^{2+}$-growth medium buffered with 25 mM HEPES; assays were then initiated by applying a fresh 1.0-ml aliquot of $^{45}Ca^{2+}$-supplemented and HEPES-buffered growth medium containing specified agonist concentrations for indicated time intervals. Assays were terminated by rapidly washing cultures with four 3-ml aliquots of Mg^{2+}-free PB containing 5 mM $LaCl_3$, and $^{45}Ca^{2+}$ content in the monolayers was expressed relative to unstimulated control cultures (6). This procedure offers the advantage of maintaining uniform $^{45}Ca^{2+}$ specific radioactivity in all $^{45}Ca^{2+}$-supplemented growth and assay media, while preserving the composition and pH of the extracellular milieu during radioisotopic equilibrium procedures.

In experiments measuring unidirectional $^{45}Ca^{2+}$ efflux following intervals of agonist exposure, BC3H-1 cultures were incubated for 30 min at 37° with specified agonist concentrations at radiosiotopic equilibrium as just described. Unidirectional efflux responses were measured by rapidly washing the culture with three 3-ml aliquots of PB and immediately applying a 2-ml aliquot of PB containing the specified agonist concentration for a 3-min efflux interval.

All assays were terminated by rapidly washing cultures with four 3-ml aliquots of Mg^{2+}-free PB containing 5 mM $LaCl_3$ (6). Data were calculated and analyzed as described in previous studies (6, 7).

Unidirectional $^{45}Ca^{2+}$ influx was measured by modification of a previous procedure (6). Cultures were rinsed free of growth medium with PB and equilibrated with a further 1-ml aliquot of PB for 10 min on a 37° bath. Influx of $^{45}Ca^{2+}$ was initiated by applying an additional 1-ml aliquot of PB containing $^{45}Ca^{2+}$ (10 μCi/ml) and indicated agonist concentrations to the culture dish with gentle swirling to ensure complete mixing. Following specified time intervals, $^{45}Ca^{2+}$ uptake was terminated in the usual manner. Uptake data were corrected for rapid and agonist-independent $^{45}Ca^{2+}$ influx measured over an initial 30-sec interval. This $^{45}Ca^{2+}$ uptake component accounted for ~25% of exchangeable cellular $^{45}Ca^{2+}$. The corrected data were calculated as an exponential approach to radioisotopic equilibrium (6), where the equilibrium uptake value was also corrected for nonspecific $^{45}Ca^{2+}$ uptake.

Concentrated drug stock solutions were freshly prepared in 10^{-2}–10^{-3} N HCl. Catecholamine oxidation in physiological buffers and growth media was prevented by addition of catalase and superoxide dismutase, each at 10 μg/ml, to all solutions (10). Assay solutions containing histamine were made by dilution from the acidic stock solution immediately before use in order to minimize decomposition.

Experimental determinations were routinely performed on duplicate or triplicate sister BC3H-1 cultures. The data shown represent mean values ± standard error compiled from replicate experiments. The number of replicate experiments, *n*, appears in parentheses accompanying each figure legend. Kinetic parameters of unidirectional $^{45}Ca^{2+}$ fluxes were estimated by fitting time course data to multiexponential processes as described previously (6).

Results

Kinetics of agonist-elicited unidirectional $^{45}Ca^{2+}$ efflux. Application of 10^{-4} M histamine to BC3H-1 cultures equilibrated with tracer $^{45}Ca^{2+}$ substantially elevates unidirectional $^{45}Ca^{2+}$ efflux, as shown in Fig. 1. Approximately 75% of the cellular $^{45}Ca^{2+}$ is available for exchange over a 30-min histamine exposure interval relative to 30% of this Ca^{2+} compartment in the absence of agonist. Previous studies of BC3H-1 cells have shown that activation of α_1-adrenergic receptors by the agonist, phenylephrine, elevates unidirectional $^{45}Ca^{2+}$ efflux (6). This observation is confirmed for the physiological agonist, norepinephrine, also shown in Fig. 1. Comparison of the responses to maximally effective concentrations of the two agonists reveals that histamine-elicited $^{45}Ca^{2+}$ efflux is comparable both in initial rate and overall extent with the response to α_1-receptor activation. Moreover, when the two agonists are added in concert, no further augmentation of $^{45}Ca^{2+}$ efflux is

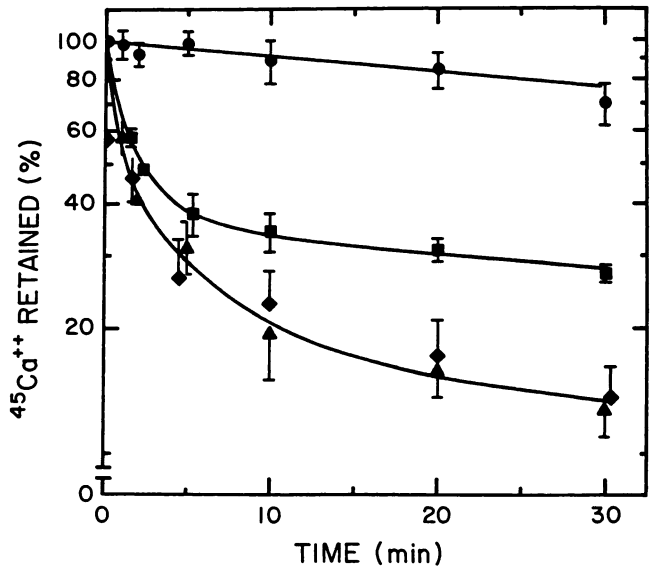

Fig. 1. Kinetics of unidirectional $^{45}Ca^{2+}$ efflux elicited by histamine and norepinephrine. BC3H-1 cell cultures were equilibrated overnight with $^{45}Ca^{2+}$-supplemented growth medium. Rapidly exchangeable $^{45}Ca^{2+}$ was removed during a 10-min incubation with PB without added $^{45}Ca^{2+}$, and agonist-stimulated efflux was monitored over indicated times thereafter (see Experimental Procedures). Data are normalized to cellular $^{45}Ca^{2+}$ content in controls which received only the initial 10-min incubation in PB ($n = 3$). ●, unstimulated efflux into PB containing no further additions; ■, histamine, 100 μM; ▲, norepinephrine, 6 μM; ♦, combined addition of histamine plus norepinephrine.

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TABLE 1

Kinetic parameters for agonist-elicited unidirectional $^{45}Ca^{2+}$ efflux in BC3H-1 cells

Estimated kinetic parameters (±SD) were derived from data in designated figures as described under Experimental Procedures.

Figure	Ligand	Unidirectional efflux			
		λ_1	k_1 (min^{-1})	λ_2	k_2 (min^{-1})
1	None	–[a]	–	0.99 ± 0.02	$10.1 \pm 1.6 \times 10^{-3}$
1	Norepinephrine, 6 μM	0.67 ± 0.05	0.94 ± 0.15	0.33 ± 0.04	$36.9 \pm 9.6 \times 10^{-3}$
1	Histamine, 100 μM	0.59 ± 0.03	1.10 ± 0.12	0.41 ± 0.02	$14.0 \pm 3.0 \times 10^{-3}$
1	Norepinephrine, 6 μM + histamine, 100 μM	0.69 ± 0.05	0.80 ± 0.12	0.31 ± 0.04	$28.4 \pm 9.3 \times 10^{-3}$

[a] –, data were fit to monophasic exponential kinetics.

observed relative to that seen with norepinephrine alone. Kinetic parameters derived from these data are collected in Table 1.

Concentration dependence of histamine-elicited $^{45}Ca^{2+}$ unidirectional efflux. The concentration dependence of agonist-elicited $^{45}Ca^{2+}$ unidirectional efflux has been quantitated from responses over an initial 3-min interval as shown in Fig. 2 (7). The data shown here were normalized relative to a maximally effective concentration of phenylephrine (10 μM), again indicating that histamine-elicited responses are comparable to those observed upon α_1-receptor activation. Analysis of these data according to the empirical Hill equation (7) reveals $K_{act} = 5.50 \pm 0.05$ μM and $n_H = 0.94 \pm 0.04$. The $^{45}Ca^{2+}$ efflux data shown in Figs. 1 and 2 were obtained following an initial 10-min incubation in PB in order to remove rapidly exchanging cellular $^{45}Ca^{2+}$ and thus to examine selectively the actions of histamine on the major fraction of slowly exchanging cellular Ca^{2+}. It is noteworthy that a similar concentration dependence for histamine-elicited $^{45}Ca^{2+}$ efflux and a similar fraction of histamine-sensitive cellular $^{45}Ca^{2+}$ relative to norepinephrine-sensitive cellular $^{45}Ca^{2+}$ were observed whether or not rapidly exchanging $^{45}Ca^{2+}$ had been previously removed (data not shown).

Inhibition of histamine-elicited responses by histamine receptor subtype-selective antagonists. Histamine receptors have been categorized as H1- or H2-subtypes (11, 12).

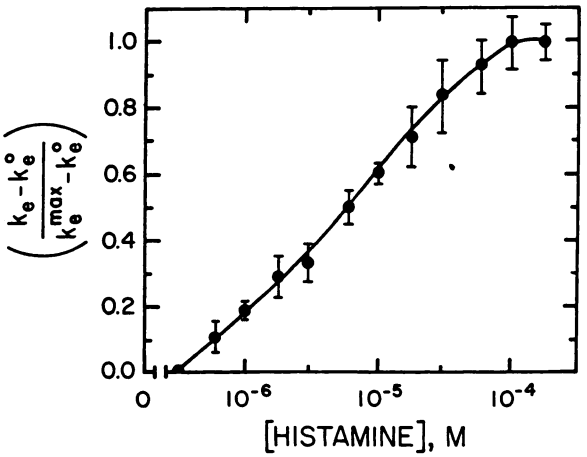

Fig. 2. Concentration dependence of the initial rate of histamine-stimulated $^{45}Ca^{2+}$ efflux. BC3H-1 cultures were equilibrated with $^{45}Ca^{2+}$ and rapidly exchangeable isotope was removed as described in Fig. 1. Agonist-elicited unidirectional $^{45}Ca^{2+}$ efflux was measured in the presence of the specified histamine concentrations over a 3-min interval. Efflux rate constants (k_e) were corrected for basal efflux (k_e^o) and normalized relative to efflux (k_e^{max}) stimulated by a maximally effective concentration of the α-adrenergic agonist phenylephrine (10 μM) assayed in sister cultures ($n = 4$).

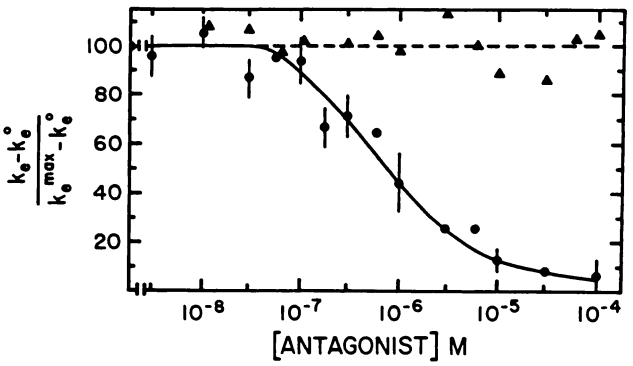

Fig. 3. Inhibition of histamine-stimulated unidirectional $^{45}Ca^{2+}$ efflux by H1 versus H2 receptor-selective antagonists. BC3H-1 cultures were equilibrated with $^{45}Ca^{2+}$ and rapidly exchangeable $^{45}Ca^{2+}$ was removed during a 10-min incubation in PB containing the specified antagonist concentrations. Inhibition of the initial rate of $^{45}Ca^{2+}$ efflux elicited by 18 μM histamine in the presence of the specified antagonist concentrations was measured over a 3-min interval and data were calculated as described in Experimental Procedures. Data for diphenhydramine (●) represent mean ± SE from three experiments. Data for cimetidine (▲) represent mean values compiled from two experiments.

H1-receptors couple to elevations of intracellular Ca^{2+} as a proximal functional response, whereas H2-receptors are linked to the adenylate cyclase system (13). It was therefore of interest to characterize the histamine receptor subtype subserving the $^{45}Ca^{2+}$ efflux response using subtype-selective antagonists. As shown in Fig. 3, the H1-receptor-selective antagonist diphenhydramine effectively antagonized $^{45}Ca^{2+}$ unidirectional efflux elicited by histamine (18 μM). Analysis of these data assuming reversible competitive inhibition (14) results in an $IC_{50} = 1.01 \pm 0.10$ μM ($n_H = 0.83 \pm 0.11$), in good agreement with values observed for diphenhydramine inhibition of histamine responses in other systems (15). In contrast, the H2-receptor-selective antagonist, cimetidine, had no effect on histamine-stimulated $^{45}Ca^{2+}$ efflux over the concentration range tested (1 nM–10 μM). In additional control experiments, the $^{45}Ca^{2+}$ efflux elicited by histamine was not affected by the α_1-adrenergic antagonist prazosin at a concentration (5.75 nM) which completely abolished the actions of norepinephrine. Conversely, diphenhydramine (10 μM) effectively inhibited histamine-stimulated $^{45}Ca^{2+}$ efflux but had no effect on norepinephrine-stimulated $^{45}Ca^{2+}$ efflux (data not shown). These data clearly indicate that histamine-stimulated Ca^{2+} efflux in BC3H-1 cells is mediated by a functional H1-histamine receptor.

Activation of unidirectional $^{45}Ca^{2+}$ influx by histamine. Activation of α_1-adrenergic receptors on BC3H-1 cells by the agonist phenylephrine mobilizes intracellular Ca^{2+}, leading to elevated unidirectional $^{45}Ca^{2+}$ efflux with a smaller increase in transmembrane $^{45}Ca^{2+}$ permeability (6). It was

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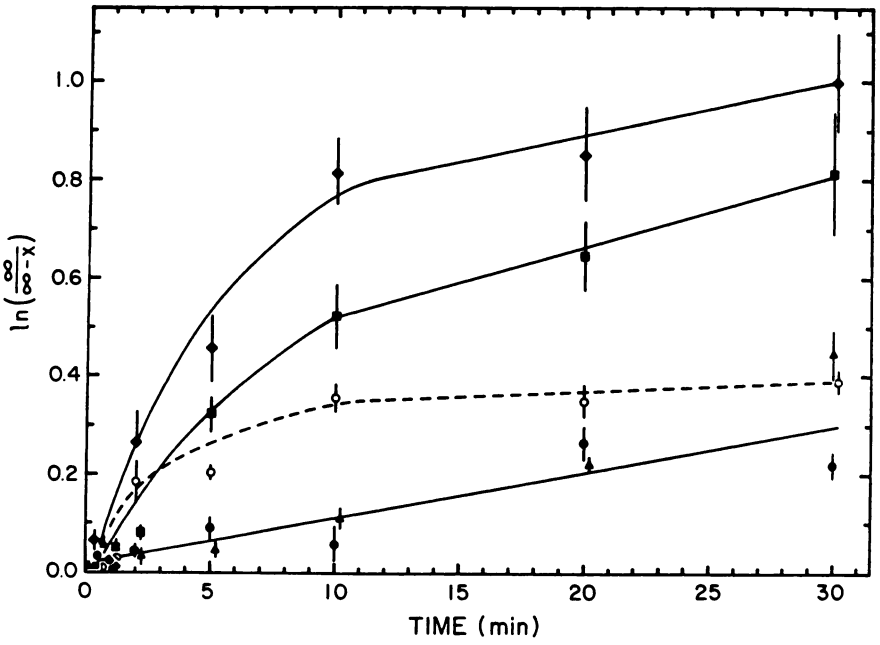

Fig. 4. Kinetics of concentration-dependent agonist-stimulated unidirectional $^{45}Ca^{2+}$ influx. BC3H-1 cultures were washed free of growth medium and equilibrated for 10 min in PB in a 37° bath. Unidirectional $^{45}Ca^{2+}$ influx was measured over the indicated time intervals in PB containing specified agonist concentrations, and data were analyzed as an exponential approach to radioisotopic equilibrium, as described in Experimental Procedures. ●, unstimulated $^{45}Ca^{2+}$ influx; ▲, 1 μM histamine; ■, 10 μM histamine; ♦, 100 μM histamine; ○, 6 μM norepinephrine. Data for histamine represent mean ± SE from seven experiments. Data for norepinephrine represent mean ± SE from four experiments.

therefore of interest to examine the influence of H1-histamine receptor activation on $^{45}Ca^{2+}$ influx. Data were corrected for the rapid initial phase of $^{45}Ca^{2+}$ uptake in order to quantitate more accurately the kinetics of agonist-stimulated $^{45}Ca^{2+}$ influx (see Experimental Procedures for details). This initial phase accounts for ~25% of exchangeable $^{45}Ca^{2+}$ uptake, occurs independently of agonist stimulation, and presumably reflects $^{45}Ca^{2+}$ adsorption to superficial sites in the monolayer. Figure 4 shows that concentrations of histamine which promote $^{45}Ca^{2+}$ efflux also substantially enhance $^{45}Ca^{2+}$ entry into BC3H-1 cells. Increasing concentrations of histamine enhance the initial rate of $^{45}Ca^{2+}$ influx as well as the fraction of cellular $^{45}Ca^{2+}$ uptake which occurs at the elevated rate. In a control experiment, histamine-stimulated $^{45}Ca^{2+}$ influx was inhibited by the H1-antagonist diphenhydramine but not by the H2-antagonist cimetidine (data not shown). For comparison, Fig. 4 also shows the $^{45}Ca^{2+}$ influx response to a maximally effective norepinephrine concentration (6 μM). Although the α-agonist increases the initial rate of $^{45}Ca^{2+}$ influx in a manner similar to that of histamine, the overall extent of agonist-stimulated influx appears less with norepinephrine than with histamine. These data measure $^{45}Ca^{2+}$ influx as an exponential approach to radioisotopic equilibrium and thus the actual rate constants for influx may be affected by alterations in the sizes of agonist-sensitive compartments resulting from receptor activation.

α_1-Adrenergic and H1-histamine receptor regulation of cellular $^{45}Ca^{2+}$ content at $^{45}Ca^{2+}$ radioisotopic equilibrium. The predominance of unidirectional $^{45}Ca^{2+}$ efflux relative to influx which is stimulated by α_1-adrenergic receptors leads to a net decline in cellular Ca^{2+} content (6). This decline results as sequestered intracellular Ca^{2+} mobilized by α-agonists becomes available for extrusion by plasma membrane Ca^{2+} transport systems with minimal compensating Ca^{2+} influx. In the case of H1-histamine receptor activation, agonist stimulation of unidirectional $^{45}Ca^{2+}$ efflux is accompanied by substantially enhanced unidirectional $^{45}Ca^{2+}$ influx (Figs. 1 and 4). These results could arise either from a bidirectional increase in transmembrane Ca^{2+} permeability stimulated by histamine receptor activation or, alternatively, from increased Ca^{2+} entry in combination with mobilization and extrusion of sequestered intracellular Ca^{2+}. In order to distinguish between these possibilities, we have compared the effects of maximal concentrations of histamine or norepinephrine upon cellular Ca^{2+} content measured using $^{45}Ca^{2+}$ radioisotopic equilibrium techniques. Independent measurements of cellular Ca^{2+} content using atomic absorption spectroscopy have previously verified that the radioisotopic equilibrium method faithfully measures alterations in total cellular Ca^{2+} (6). When BC3H-1 cultures are exposed to norepinephrine (6 μM) at radioisotopic equilibrium, cellular $^{45}Ca^{2+}$ declines by ~30% relative to unstimulated controls and is maintained at this level up to 30 min in the continued presence of agonist (Fig. 5A). Similar results were obtained previously using the agonist phenylephrine (6). Activation of H1-histamine receptors by 100 μM histamine produces a rapid

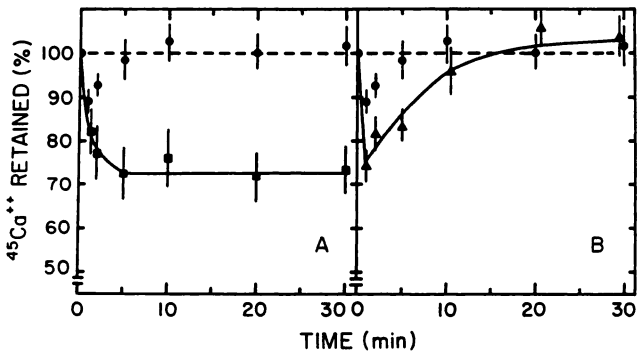

Fig. 5. The influence of α_1-adrenergic and H1-histamine receptor activation on cellular $^{45}Ca^{2+}$ content measured at radioisotopic equilibrium. BC3H-1 cultures equilibrated overnight with $^{45}Ca^{2+}$-supplemented growth medium in a controlled atmosphere incubator were transferred into fresh solutions of $^{45}Ca^{2+}$-supplemented growth medium and incubated under room atmosphere at 37° for an initial 10-min interval. This solution was then replaced by fresh medium containing $^{45}Ca^{2+}$ at the identical specific radioactivity plus indicated concentrations of agonist. Cellular $^{45}Ca^{2+}$ content was measured following the indicated time intervals and data were normalized relative to cultures which received only the initial 10-min incubation interval (n = 5). ●, no agonist additions; ■, norepinephrine, 6 μM (A); ▲, histamine, 100 μM (B).

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but transient decline in cellular $^{45}Ca^{2+}$ content relative to unstimulated controls which subsequently returns to basal values despite the continued presence of agonist (Fig. 5B). These data indicate that H1-receptors mediate both mobilization of sequestered intracellular Ca^{2+} and Ca^{2+} entry into the cell. If the enhanced unidirectional $^{45}Ca^{2+}$ fluxes activated by histamine had resulted exclusively from enhanced transmembrane permeability, then one would expect a net increase in cellular Ca^{2+} content, reflecting Ca^{2+} movement down its inwardly directed concentration gradient. Thus, activation of both H1-histamine receptors and α_1-adrenergic receptors on BC3H-1 cells mobilizes Ca^{2+} from common intracellular stores. Activation of H1 receptors elicits an appreciable additional component of Ca^{2+} influx, so that cellular Ca^{2+} content declines transiently and then recovers during the continued presence of histamine.

The influence of prior agonist exposure upon receptor response. Having identified functional H1-histamine receptors that mobilize intracellular Ca^{2+} from a source similar to that accessed by α_1-receptor activation, we turned to examine the determinants of agonist-induced alterations in cellular responsiveness. Results are shown in Fig. 6. BC3H-1 cultures were incubated for 30 min at $^{45}Ca^{2+}$ radioisotopic equilibrium with specified concentrations of norepinephrine, resulting in a concentration-dependent depletion of cellular $^{45}Ca^{2+}$ content (Fig. 6A) as expected from the data in Fig. 5A. Following this

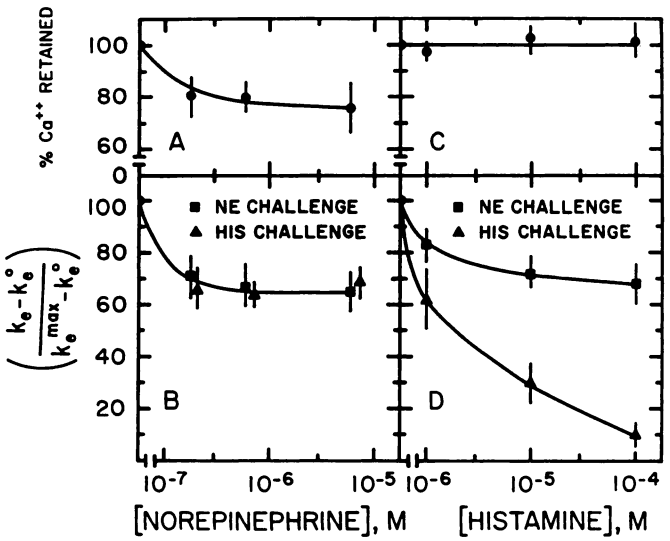

Fig. 6. The influence of prior agonist exposure on the disposition of cellular $^{45}Ca^{2+}$ and receptor functional responsivenes. Sets of cultures equilibrated overnight with $^{45}Ca^{2+}$-supplemented growth medium were incubated for 30 min with specified agonist concentrations at $^{45}Ca^{2+}$ radioisotopic equilibrium, using the transfer protocol described under Experimental Procedures. Following this 30-min interval, cultures were analyzed in triplicate for cellular $^{45}Ca^{2+}$ content (●, A, C) or for $^{45}Ca^{2+}$ unidirectional efflux responses (B, D) in PB containing maximally effective concentrations of either norepinephrine (6 μM, ■) or histamine (100 μM, ▲). Unidirectional $^{45}Ca^{2+}$ efflux was measured by washing away the conditioning solution with three 3-ml aliquots of PB, allowing efflux to proceed for 3 min into a 2-ml aliquot of PB containing the specified agonist concentration, and terminating the reaction by washing the culture with four 3-ml aliquots of PB containing $LaCl_3$. Cellular $^{45}Ca^{2+}$ content was measured by terminating the conditioning interval with four 3-ml aliquots of PB containing $LaCl_3$ without intervening efflux. Efflux data (k_e) were corrected for basal efflux (k_e^o) and normalized to maximum response values (k_e^{max}) obtained in sets of control cultures which received no added agonist during the 30-min incubation at isotopic equilibrium (n = 6).

conditioning interval, cultures were challenged with maximally effective concentrations of either norepinephrine or histamine and unidirectional $^{45}Ca^{2+}$ efflux responses were measured over an initial 3-min interval (Fig. 6B). Data were normalized relative to responses obtained from control cultures which received no agonist exposure during the conditioning interval. As shown in Fig. 6B, prior norepinephrine exposure reduces the response to subsequent challenge by either histamine or norepinephrine to similar extents (~70% of naive controls). This result suggests that the observed decrement in response occurs distally to the α_1-receptor at some point common to both α_1-adrenergic and H1-histamine receptor coupling to intracellular Ca^{2+} mobilization. The complementary experiment was performed by measuring cellular responses following exposure to histamine. As shown in Fig. 6C, 30 min incubation of cultures with indicated histamine concentrations at $^{45}Ca^{2+}$ radioisotopic equilibrium does not alter cellular $^{45}Ca^{2+}$ content relative to unstimulated controls. This result is consistent with the data in Fig. 5B which show the decline in cell Ca^{2+} content elicited by histamine is transient and recovers to basal levels during this time interval. When cultures previously conditioned with histamine are challenged by maximal concentrations of either norepinephrine or histamine in unidirectional $^{45}Ca^{2+}$ efflux measurements (Fig. 6D), responses to norepinephrine are again reduced to ~70% of control, complementary to the results observed following norepinephrine conditioning. Strikingly, prior exposure to specified histamine concentrations progressively and completely abolishes the $^{45}Ca^{2+}$ efflux response to a subsequent histamine challenge. This result suggests that histamine exposure selectively converts the H1-histamine receptor to a refractory or densensitized state.

The influence of prior agonist exposure upon the concentration dependence of agonist-elicited $^{45}Ca^{2+}$ efflux. In order to characterize further the influence of agonist exposure upon receptor responsiveness, we examined the concentration dependence for receptor activation of $^{45}Ca^{2+}$ efflux following 30 min conditioning with specified agonist concentrations at $^{45}Ca^{2+}$ radioisotopic equilibrium. Estimates of efflux values (k_e) obtained following exposure to a given conditioning agonist concentration were corrected for the unstimulated efflux (k_e^o) measured following that same conditioning agonist concentration. Results are presented in Fig. 7. In the case of norepinephrine exposure (Fig. 7A), the decrement in α_1-adrenergic receptor response observed previously (Fig. 6B) results principally from a decrease in the maximum obtainable response without a substantial alteration in the concentration dependence for norepinephrine activation of $^{45}Ca^{2+}$ efflux. Consideration of the primary data from which Fig. 7A was calculated reveals a further aspect of the influence of norepinephrine upon cellular $^{45}Ca^{2+}$ mobility, as shown in Fig. 7B. That is, conditioning cells with norepinephrine elevates unstimulated $^{45}Ca^{2+}$ efflux in addition to decreasing the maximal agonist-stimulated response. By contrast, incubation of cultures with fixed concentrations of histamine results in a progressive increase in the concentrations of histamine subsequently required to activate $^{45}Ca^{2+}$ efflux (Fig. 7C). We also note that prior exposure to histamine has no effect on subsequent measurements of unstimulated $^{45}Ca^{2+}$ efflux. Thus, the loss in histamine receptor responsiveness induced by exposure to histamine reflects a decrease in the apparent affinity of the receptor for agonist.

Kinetics of $^{45}Ca^{2+}$ efflux following agonist exposure.

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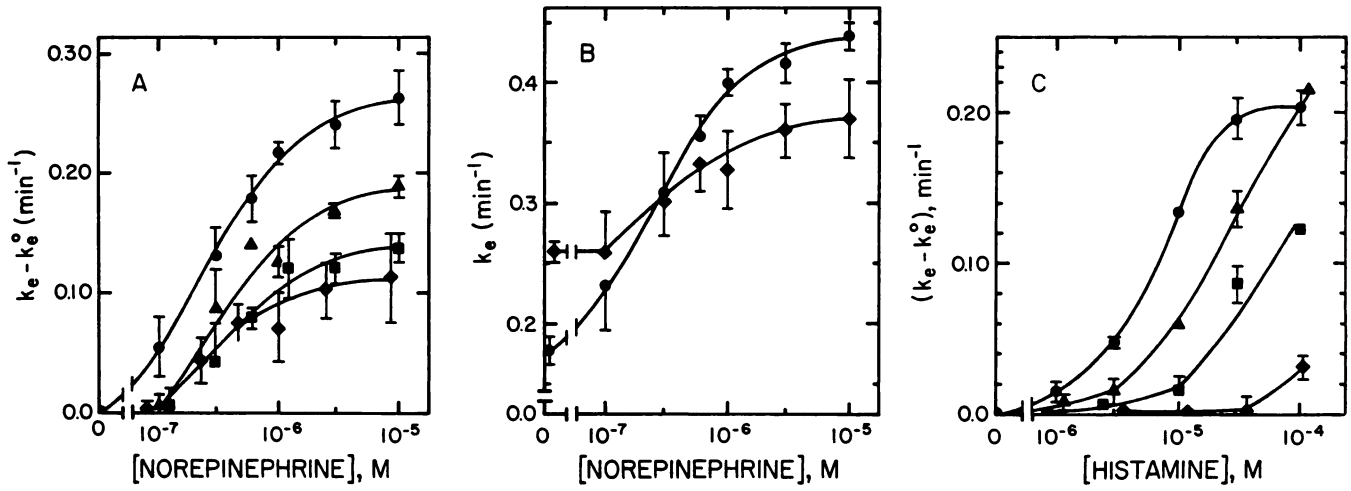

Fig. 7. The influence of prior agonist exposure upon the concentration dependence for agonist-elicited unidirectional $^{45}Ca^{2+}$ efflux. Sets of BC3H-1 cultures were incubated for 30 min at $^{45}Ca^{2+}$ radioisotopic equilibrium with fixed agonist concentrations. Following this conditioning interval, the solution was rapidly washed off and replaced with a fresh solution of PB containing specified agonist concentrations. Unidirectional $^{45}Ca^{2+}$ efflux was measured over a 3-min interval and calculated relative to cellular $^{45}Ca^{2+}$ content measured following conditioning in the presence of the specified agonist concentration as described in Fig. 6. A. Prior exposure to norepinephrine (n = 4): ●, no agonist preexposure; ▲, norepinephrine, 0.18 μM; ■, 0.60 μM; ♦, 6.0 μM. Experimentally determined efflux rate constants (k_e) were corrected for unstimulated efflux (k_e^o) determined at the corresponding norepinephrine conditioning concentration. B. Prior exposure to norepinephrine. Agonist-stimulated efflux rate constants (k_e) obtained in A following conditioning with no added agonist (●) or 6 μM norepinephrine (♦) are presented without correction for unstimulated efflux. C. Prior exposure to histamine (n = 3). ●, no agonist preexposure; ▲, histamine, 1 μM; ■, 10 μM; ♦, 100 μM. In this experiment unstimulated efflux rate constants obtained following exposure to agonist were not significantly different from those obtained in the absence of agonist preexposure.

Since the data shown in Fig. 7B indicated that cellular Ca^{2+} mobility was altered following agonist exposure, it was of interest to examine in greater detail the initial kinetics of $^{45}Ca^{2+}$ efflux following 30 min agonist conditioning at radioisotopic equilibrium. Data were normalized to cellular $^{45}Ca^{2+}$ content following the appropriate exposure condition in order to determine accurately relative rates of $^{45}Ca^{2+}$ efflux. As shown in Fig. 8A, unstimulated $^{45}Ca^{2+}$ efflux following removal of the conditioning norepinephrine solution remained elevated over an initial segment of approximately 1 min before returning to values seen in controls which had received no agonist conditioning. It should be noted that this result was obtained despite the presence of the antagonist phentolamine (10 μM) in all wash and assay solutions used to replace the conditioning norepinephrine solution. We assume that under these conditions receptor occupation by agonist should be rapidly terminated although the possibility of continuing receptor stimulation by slowly dissociating agonist cannot be excluded. Fig. 8A also shows that the efflux response elicited by a maximally effective norepinephrine concentration following norepinephrine exposure remains essentially intact relative to naive controls.

The complementary set of experiments was performed using histamine as shown in Fig. 8B. Consistent with previous results, unstimulated $^{45}Ca^{2+}$ efflux following histamine exposure was identical to unstimulated $^{45}Ca^{2+}$ efflux from control cultures. Furthermore, cultures previously conditioned with a saturating histamine concentration (100 μM) at radioisotopic equilibrium and subsequently challenged with histamine showed $^{45}Ca^{2+}$ efflux kinetics identical to that of unstimulated cultures. The efflux response to histamine challenge in cultures which received no prior agonist is shown for comparison and emphasizes the refractoriness of the histamine response following histamine exposure.

Discussion

H1-histamine receptor control of cellular Ca^{2+}. Receptors which mediate vascular smooth muscle contraction may either utilize activator Ca^{2+} present within sequestered intracellular stores or enhance Ca^{2+} entry across the plasma membrane (1). We have monitored $^{45}Ca^{2+}$ radioisotopic tracer movements in order to characterize mechanisms of H1-histamine receptor control of intracellular Ca^{2+} in BC3H-1 cells. Over its effective concentration range, histamine promotes both unidirectional $^{45}Ca^{2+}$ efflux and influx. The receptor has been identified as the H1-subtype, as has been the case for histamine receptors in proximal relationship to cellular Ca^{2+} regulation (13). The histamine-stimulated unidirectional $^{45}Ca^{2+}$ efflux response has been quantitated, and the agonist concentration dependence correlates well with that observed previously for histamine-induced contraction in vascular smooth muscle (16, 17), vas deferens (18), and airway smooth muscle (19). The transmembrane Ca^{2+} movements elicited by histamine result in an initial transient decrease in cellular Ca^{2+} content followed by a subsequent recovery to basal values. These data indicate that histamine receptor activation elevates cytoplasmic Ca^{2+} concentration both by mobilizing sequestered intracellular Ca^{2+} and by increasing transmembrane Ca^{2+} permeability. This behavior stands in contrast to previous observations of regulation of cellular Ca^{2+} by α_1-adrenergic receptors or nicotinic acetylcholine receptors on sister clones of BC3H-1 cells. The nicotinic receptor represents the prototypical receptor-operated channel where agonist activation leads to a bidirectional increase in transmembrane permeability to small cations (20). Activation of nicotinic acetylcholine receptors in BC3H-1 cells increases unidirectional $^{45}Ca^{2+}$ influx more than 100-fold, whereas unidirectional $^{45}Ca^{2+}$ efflux is elevated less than 2-fold (6). Enhancing transmembrane Ca^{2+} permeability in the presence of the large, inwardly directed Ca^{2+} concentration gradient leads to an immediate and sustained increase in cellular $^{45}Ca^{2+}$ con-

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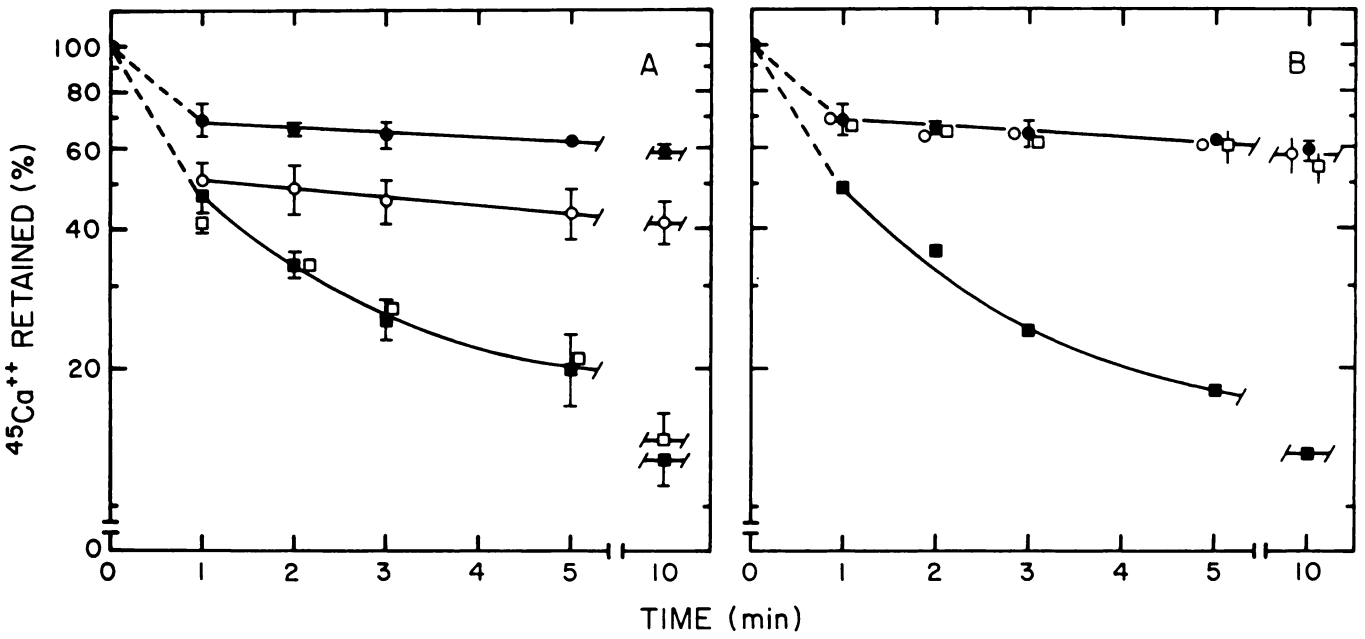

Fig. 8. Kinetics of unidirectional $^{45}Ca^{2+}$ efflux following agonist exposure at $^{45}Ca^{2+}$ radioisotopic equilibrium. BC3H-1 cultures were incubated with specified agonist concentrations for 30 min at $^{45}Ca^{2+}$ radioisotopic equilibrium. Following this interval the conditioning solution was rapidly washed away and replaced with PB containing specified agonist or antagonist concentrations for indicated times. When unidirectional efflux was measured in the presence of antagonists, these ligands were also included in the buffer used to wash out the conditioning solution. Data were calculated relative to cellular $^{45}Ca^{2+}$ content measured following the conditioning interval in the presence of the specified agonist (n = 3). The conditions for each kinetic course are described as "agonist addition during radioisotopic equilibrium → agonist addition during unidirectional efflux." A. Adrenergic responsiveness following norepinephrine exposure. ●, no agonist addition during $^{45}Ca^{2+}$ radioisotopic equilibrium → no agonist addition during unidirectional efflux (unstimulated control); ■, no added agonist → norepinephrine (6 μM); □, norepinephrine (6 μM) → norepinephrine (6 μM); ○, norepinephrine (6 μM) → phentolamine (10 μM). In these experiments, exposure to 6 μM norepinephrine for 30 min at radioisotopic equilibrium decreased cell $^{45}Ca^{2+}$ content to 81 ± 3% of control values. B. Histamine responsivenes following histamine exposure. ●, no agonist addition → no agonist addition (same data as in A); ■, no agonist addition → histamine (100 μM); □, histamine (100 μM) → histamine (100 μM); ○, histamine (100 μM) → diphenhydramine (10 μM). Exposure to histamine during the 30-min conditioning interval did not significantly alter cellular $^{45}Ca^{2+}$ content relative to unstimulated controls.

tent at radioisotopic equilibrium. Activation of α_1-adrenergic receptors produces Ca^{2+} movements consistent with the predominant response of intracellular Ca^{2+} mobilization. That is, unidirectional $^{45}Ca^{2+}$ efflux is selectively enhanced with smaller effects on unidirectional influx. A net 30–40% decline in cellular Ca^{2+} content results as sequestered intracellular Ca^{2+} is mobilized and made accessible to transmembrane extrusion. By contrast, the H1-receptor identified in these cells appears to possess a complex linkage capable of eliciting substantial mobilization of intracellular Ca^{2+} as well as transmembrane Ca^{2+} entry.

In addition, we note that activation of both H1- and α_1-receptors mobilizes intracellular Ca^{2+} from a similar source. This conclusion is supported by data in Fig. 1, which show that maximally effective concentrations of histamine or norepinephrine, either alone or in concert, elicit comparable $^{45}Ca^{2+}$ efflux responses. The kinetics and relative extents of these efflux responses are maintained in Ca^{2+}-free PB, further arguing that the two receptors access similar fractions of intracellular Ca^{2+}. These data are summarized in Table 2.

The influence of prior agonist exposure upon receptor responses. The presence of two independent receptor types which mobilize Ca^{2+} from common intracellular stores has been utilized by conditioning cells with specified agonist concentrations and then monitoring unidirectional $^{45}Ca^{2+}$ efflux as a measure of Ca^{2+} mobilization in response to a subsequent challenge by either the homologous or heterologous agonist. This paradigm allows delineation of the relative contributions of altered intracellular Ca^{2+} disposition versus receptor refractoriness to the decreased cellular responses which are observed following prolonged agonist exposure. A similar strategy was employed to examine refractoriness of the response of adenylate cyclase to catecholamines versus prostaglandins (21). Continued activation by the α-adrenergic agonist in our system results in a parallel decrement in response to subsequent challenges by either histamine or norepinephrine (Fig. 6B). The reduction in α_1-adrenergic response following norepinephrine conditioning results primarily from a decrease in the maximum obtainable response without alteration in the concentration dependence for agonist activation (Fig. 7A). Thus, the α_1-adrenergic receptor maintains its ability to mobilize intracellular Ca^{2+} substantially intact, and the reductions observed in functional response should occur at some point common to both α_1- and H1-receptor-effector coupling. Specifically, the predominant effect of sustained α_1-receptor activation observed in these studies is an alteration in the disposition of cellular Ca^{2+} toward a new steady state. Total cellular stores of agonist-sensitive Ca^{2+}, although reduced (Figs. 5A and 6A), remain sufficient to fuel appreciable unidirectional $^{45}Ca^{2+}$ efflux responses (Figs. 6B, 7A, and 8A). At the same time, incubation with α-agonist elicits a sustained increase in the availability of cellular Ca^{2+} stores to transmembrane efflux which is evident even after the agonist is removed (Figs. 7B and 8A). These results present an apparent paradox: in the presence of agonist the cell remains

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TABLE 2

Kinetic parameters for unidirectional $^{45}Ca^{2+}$ efflux in normal and Ca^{2+}-free physiological buffer

Estimated kinetic parameters (±SD) were calculated as described under Experimental Procedures.

External [$CaCl_2$]	Ligand	λ_1	k_1 (min^{-1})	λ_2	k_2 (min^{-1})
mM					
1.8	None	–[a]	–	0.97 ± 0.02	$11.2 \pm 1.4 \times 10^{-3}$
1.8	Norepinephrine, 6 μM	0.74 ± 0.08	0.67 ± 0.15	0.26 ± 0.07	$11.0 \pm 1.4 \times 10^{-3}$
1.8	Histamine, 100 μM	0.63 ± 0.06	0.66 ± 0.14	0.38 ± 0.05	$12.3 \pm 7.6 \times 10^{-3}$
~0[b]	None	–	–	0.97 ± 0.02	$8.8 \pm 1.3 \times 10^{-3}$
~0[b]	Norepinephrine, 6 μM	0.58 ± 0.02	1.05 ± 0.08	0.42 ± 0.02	$18.6 \pm 2.3 \times 10^{-3}$
~0[b]	Histamine, 100 μM	0.55 ± 0.02	1.21 ± 0.14	0.45 ± 0.02	$10.4 \pm 2.5 \times 10^{-3}$

[a] –, data were fit to monophasic exponential kinetics.

[b] Cultures equilibrated with $^{45}Ca^{2+}$ were subjected to an initial 10-min preincubation with PB in the usual manner to remove rapidly exchanging $^{45}Ca^{2+}$. Extracellular Ca^{2+} was removed by rapidly washing the cultures with three 3-ml aliquots of Ca^{2+}-free PB containing 5 mM EGTA, and a 2-ml aliquot of Ca^{2+}-free PB containing specified ligands was applied for the same efflux time intervals employed in Fig. 1. Assays were terminated as usual.

continuously able to mobilize intracellular Ca^{2+}, yet sensitive intracellular Ca^{2+} stores remain largely intact. Although inferential in nature, these data strongly suggest that cellular Ca^{2+} movements between mobile and sequestered intracellular compartments are related in a dynamic and reversible cycle. This concept has been elegantly reviewed by Rasmussen and Barrett (22). In theory, agonist-sensitive intracellular Ca^{2+} stores could be replenished by compensating Ca^{2+} influx across the plasma membrane or by continued recycling of Ca^{2+} between cytoplasm and agonist-sensitive intracellular stores. Fig. 4 shows that α-adrenergic stimulation does in fact increase transmembrane Ca^{2+} entry, whereas measurements of free intracellular Ca^{2+} in BC3H-1 monolayers provide evidence for cycling of Ca^{2+} by intracellular organelles (23).

Recent data from hepatocytes (24) and BC3H-1 cells (25) suggest that the α_1-receptor may exhibit multiple states of affinity for agonist which can be discriminated at reduced temperature or in the presence of guanine nucleotides. Our findings indicate that, if such interconversion occurs under physiological conditions in BC3H-1 cells, the transition between states must be rapid relative to the 3-min duration of the $^{45}Ca^{2+}$ efflux measurement. Furthermore, the equilibrium distribution of receptor states achieved following 30 min exposure to agonist should retain a substantial proportion of responsive receptor molecules relative to naive controls. In additional experiments, α_1-agonist affinity has been measured by competition with initial rates of [^{3}H]prazosin binding to intact cells over a 20-sec interval at 37°. Prior exposure of the receptor to agonist results in a small (≤3-fold) decrease in apparent agonist affinity compared to cultures simultaneously exposed to agonist plus [^{3}H]prazosin, further arguing against the presence of substantial agonist-induced alterations in α_1-adrenergic receptor state.[1] Moreover, the relatively brief periods of agonist exposure employed in the present study do not result in significant α_1-receptor down-regulation.[2]

Prior histamine exposure results in decreased response upon challenge by norepinephrine (Fig. 6D), in parallel with the diminished response elicited by histamine following norepinephrine conditioning (Fig. 6B). This reduction in response to a heterologous agonist following histamine exposure occurs despite the apparent recovery of total cellular Ca^{2+} content (Fig. 6C) and unstimulated efflux rate constants (Fig. 8B) to resting values, suggesting that the disposition of agonist-sensitive intracellular Ca^{2+} has been altered or that additional coupling steps have been perturbed. Conversely, the selective and differential inhibition of H1-receptor response observed following exposure to histamine appears to represent an example of agonist-induced receptor desensitization. Conditioning cultures with histamine decreases the apparent affinity with which a subsequent histamine challenge activates $^{45}Ca^{2+}$ efflux (Fig. 7C). The altered histamine concentration dependence could result from temporary inactivation of an initial receptor reserve, leading to a reduction in apparent agonist potency. Examples of tachyphylaxis upon prolonged or repetitive application of histamine have been noted previously in intact smooth muscle preparations (16), and the BC3H-1 system offers the opportunity to study further the molecular basis of this phenomenon.

The results from the BC3H-1 system may be compared with recent data from aortic smooth muscle. Lurie *et al.* (26) found that 7 hr exposure of intact aortic rings to either norepinephrine or histamine resulted in homologous desensitization of the contractile response for each agonist. No alteration was observed in the number of α_1-receptors detected in membrane homogenates prepared from the tissue. Agonist-induced desensitization was reflected by decreased agonist potency in eliciting contractions, similar to the results we have obtained with histamine. Additional measurements of α_1-receptor-stimulated phosphatidylinositol synthesis following 7 hr norepinephrine exposure revealed decreased responsiveness consistent with the $^{45}Ca^{2+}$ efflux data in our study. That is, the maximum response obtained decreased ~30% without alteration in the concentration dependence for norepinephrine activation. These variations in α_1-receptor response following agonist exposure may relate to nonequivalent coupling mechanisms between receptor occupancy and component cellular responses, to differences in the cell of origin, or to the differentiated phenotypes of these divergent experimental systems.

The $^{45}Ca^{2+}$ flux experiments presented here provide only an indirect reflection of the dynamics of intracellular Ca^{2+}. Direct measurements of intracellular free Ca^{2+} using Ca^{2+}-sensitive fluorescent chelators can distinguish whether alterations in Ca^{2+} efflux following agonist conditioning truly reflect cytoplasmic Ca^{2+} availability or whether the activity of the plasma membrane Ca^{2+} transport system is perturbed under these circumstances. Examination of the relationship between Ca^{2+} mobilization and phosphoinositide hydrolysis poses an addi-

[1] R. D. Brown, unpublished data.

[2] R. J. Hughes, personal communication.

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tional goal. Heterogeneities between different receptor types in the relative susceptibilities of these two responses to agonist-induced desensitization could shed further insight into their mechanistic relationships.

In sum, these studies contrast the means by which α_1-adrenergic and H1-histamine receptors regulate intracellular Ca^{2+} in BC3H-1 cells. The phasic response to histamine appears to reflect agonist-induced desensitization at the level of the receptor itself, whereas the α_1-receptor remains functionally coupled to intracellular Ca^{2+} during prolonged agonist stimulation. Ongoing receptor activation alters the disposition of intracellular Ca^{2+} by modulating the dynamic cycling of Ca^{2+} among multiple transport and storage systems. The sustained responsiveness of the α_1-receptor in this simplified cell culture system may reflect the physiological function of the receptor in steady state maintenance of vascular tone by the sympathetic nervous system.

Acknowledgments

We are indebted to Karen D. Berger for growth of BC3H-1 cell cultures and to Palmer Taylor, Kelly Ambler, Richard Hughes, and Paul Culver for numerous helpful discussions during the course of this study.

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