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Kinetic Analysis of Secretion from Permeabilized Adrenal Chromaffin Cells Reveals Distinct Components*

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Mary A. Bittner‡ and Ronald W. Holz
From the Department of Pharmacology, University of Michigan Medical School, Ann Arbor, Michigan 48109-0626

We have determined that there are components to the time course of Ca^{2+}-dependent secretion from digitonin-permeabilized bovine adrenal chromaffin cells that can be distinguished by Ca^{2+} sensitivity and ATP dependence. The effects of various Ca^{2+} concentrations are different on the initial rates and later rates of secretion. The earliest rates (5 s) are half-maximal between 30–100 μM Ca^{2+} and maximal by 300 μM Ca^{2+}. Later rates of secretion are maximal by 10 μM and decline above 30 μM Ca^{2+}. At low Ca^{2+} concentrations secretion begins after a lag of several seconds. The early rates of secretion (within 1 min) are dependent on the prior effects of MgATP. MgATP primes the cells to secrete. Later rates require the continuous presence of MgATP for optimal secretion. Incubation with low concentrations of Ca^{2+} increases the ability of MgATP to stimulate subsequent Ca^{2+}-dependent secretion. Preincubation with Ca^{2+} has no effect on the rapid loss of ATP-independent secretion with time after permeabilization. The data indicate that: 1) as secretion progresses in digitonin-permeabilized cells, different events become rate-limiting; 2) maximal secretion at the early times requires at least 10-fold higher Ca^{2+} concentrations than at later times; 3) the rate at which Ca^{2+} initiates secretion is concentration-dependent; and 4) Ca^{2+} not only triggers the final events in secretion but enhances the ability of ATP to prime secretion.

In recent years permeabilized cell models have been developed and used to probe the molecular requirements for exocytosis. Such studies have established the primary roles of Ca^{2+} and ATP (1–4), investigated the actions of guanine nucleotides and guanine nucleotide-binding proteins (5–8), explored the possible roles of various kinases, including protein kinase C (9–12), and utilized proteins such as clostridial neurotoxins to inhibit secretion (13–15). Despite the success of these studies, little is known about the ordering of steps in regulated secretion. The purpose of this study is to identify the sequence of events responsible for regulated exocytosis.

Digitonin-permeabilized chromaffin cells are highly permeable to salts and nucleotides and are leaky to proteins. Secretion is triggered by buffered Ca^{2+} concentrations in the range of 0.3–30 μM. Use of this model has revealed that secretion occurring immediately after the addition of Ca^{2+} differs in some respects from that seen some minutes after Ca^{2+}. In a previous study we demonstrated that one major difference is a requirement for ATP in the incubation medium (16). Intact cells are primed by intracellular ATP so that immediately upon permeabilization there is a component of secretion which is independent of exogenous ATP. This ATP-independent secretion is rapid and terminates after 2 min. Since it takes several minutes for the primed state to decay, a preincubation in the absence of Mg and ATP is necessary to demonstrate a substantial ATP dependence for secretion. ATP-dependent secretion occurs more slowly than ATP-independent secretion and continues at a relatively constant rate for 12 min. ATP partially maintains the primed state after permeabilization by acting before Ca^{2+} in the secretory pathway.

In this study we expand on the observation that the secretory process changes its characteristics with time after the addition of Ca^{2+}. We examine the different Ca^{2+} sensitivities of ATP-dependent and ATP-independent secretion, and we demonstrate a role for Ca^{2+} in ATP-dependent priming. The results of these experiments suggest a model for the sequence of events in the exocytotic pathway.

MATERIALS AND METHODS

Primary dissociated cells from bovine adrenal medulla were prepared and maintained as monolayer cultures in Eagle's minimal essential medium (GIBCO) containing 10% heat-inactivated fetal calf serum. Cells were usually cultured as monolayers in 6.4-mm diameter collagen-coated plastic culture wells (Costar, Cambridge, MA) at a density of 500,000 cells/cm^2. Sterile Vitrogen 100 bovine dermal collagen solution (32 μg/ml in 0.01 N HCl) (Celtrix Laboratories, Palo Alto, CA) was applied to each 6.4-mm diameter well (5 μg/cm^2) and allowed to air dry before the cells were plated. Cells were prepared and plated in the presence of 1.3 μg/ml Fungizone (Squibb, Princeton, NJ). At least 12 h prior to the start of an experiment, the culture medium was replaced by medium without Fungizone. Experiments were performed 4–8 days after the preparation of the cultures.

Immediately before a secretion experiment, cells were incubated for 3 h in culture medium containing [^{3}H]norepinephrine and 0.5 mM ascorbate. Cultures were rinsed for at least 30 min in fresh culture medium without [^{3}H]norepinephrine or with a physiological salt solution (CaPSS)[1] containing 145 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, 15 mM HEPES (pH 7.4), 0.5 mM ascorbate, and 0.5% bovine serum albumin. The potassium

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‡ To whom correspondence should be addressed: Dept. of Pharmacology, M6322 Medical Science Bldg. I, University of Michigan Medical School, Ann Arbor, MI 48109-0626. Tel.: 313-747-3127; Fax: 313-763-4450.

[1] The abbreviations used are: CaPSS calcium-containing physiological salt solution; BAPTA bis-(o-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid; DMPP dimethylphenylpiperazinium; EGTA [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KGEP potassium glutamate, EGTA, PIPES-containing solution; KGENP potassium glutamate, EGTA, NTA, PIPES-containing solution; NTA *N,N*-bis[carboxymethyl]glycine; nitrilotriacetic acid; PIPES piperazine-*N,N'*-bis-(2-ethanesulfonic acid); SLO streptolysin-O; GTPγS, guanosine 5'-(3-(thiotriphosphate).