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cAMP Modulates Exocytotic Kinetics and Increases Quantal Size in Chromaffin Cells

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ABSTRACT

The role of cAMP/cAMP-dependent protein kinase (PKA) on the late phase of exocytosis has been studied by amperometry on Ba^{2+}-stimulated single bovine chromaffin cells. Forskolin (FSK) increases the intracellular cAMP levels in a concentration-dependent manner. Forskolin (100 nM) does not increase the number of exocytotic events, although it significantly increases the net granule content of catecholamines (CA), which is accompanied by a slowing of the process of degranulation. These effects are reversible, occur within 15 to 60 s, and are not due to newly synthesized CA. Isoprenaline, pituitary adenylate cyclase-activating polypeptide-38 or dB-cAMP reproduce FSK effects as does cholera toxin. The inhibition of phosphodiesterases with 3-isobutyl-1-methylxanthine mimics and potentiates the effect of FSK and isoprenaline. Rolipram and okadaic acid also produce a drastic increase in net granule content of CA, whereas H-89 attenuates the FSK response. These data indicate that cyclic AMP/PKA might favor the granule aggregation before its fusion with cell membrane and slow the late step of the exocytotic process.

Adrenal chromaffin cells release catecholamines (CA) to the blood stream by exocytosis, a process that entails the fusion of an intracellular secretory vesicle, named chromaffin granule, to the plasma membrane. Catecholamines and other soluble components are stored within the chromaffin granule at concentrations as high as 0.5 to 1 M (Jankowski et al., 1993; Albillos et al., 1997). Several second messengers have been implicated in the trigger and modulation of CA release. However, most of the studies carried out to address the question of the role of intracellular signaling in secretion were done by measuring the total CA secreted from populations containing thousands or millions of chromaffin cells. In these studies, the effects observed in secretion were addressed to changes in the number of granules that were released and did not take into account that these vesicles could vary their CA content nor that cAMP could modify the intrinsic kinetics of exocytosis. No studies have yet been performed at single event level.

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Most authors found that cell treatment with forskolin or cAMP analogs resulted in a stimulatory effect on basal and evoked adrenomedullary secretion (Knight and Baker, 1982; Morita et al., 1987; Parramón et al., 1995; Przywara et al., 1996; Alvarez et al., 1997), but data in the opposite direction are also available [i.e., an inhibition by cAMP on CA release evoked by cholinergic agonists (Baker et al., 1985; Cheek and Burgoyne, 1987)].

In trying to find an explanation on the underlying mechanisms, cAMP was described as activating Ca^{2+} channels of chromaffin cells (Morita et al., 1987; Doupnik and Pun, 1992; Parramón et al., 1995), blocking K^+ channels (Garber et al., 1990), and modifying the cytoskeleton dynamics (Cheek and Burgoyne, 1987; Perrin et al., 1992). However, some of the effects observed with FSK concentrations over 10 μM seem to be unrelated to its ability to stimulate cAMP production (Gandía et al., 1997).

Protein kinases regulate many biological functions acting on multiple cellular processes, the exocytotic phenomenon is not an exception. Recently, we have reported that NO, acting on the PKG cascade, promoted dramatic changes in the exocytotic kinetics (Machado et al., 2000). Similar results were also found upon activation of PKC (Graham et al., 2000). These observations were made with the use of amperometry with carbon microelectrodes, which allows the direct analysis

ABBREVIATIONS: CA, catecholamines; FSK, forskolin; PKG, cGMP-dependent protein kinase; PKC, Ca^{2+}/phospholipid-dependent protein kinase; PKA, cAMP-dependent protein kinase; C-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide potassium; $t_{1/2}$, spike width at half height; Q, spike net charge; m, ascending slope of spike; tP, time to reach the spike maximum; IBMX, 3-isobutyl-1-methylxanthine; HPLC, high pressure liquid chromatography; VMAT, vesicular monoamine transporter; dB-cAMP, N^6,2′-*O*-dybutyril-3′:5′-cyclic monophosphate; PACAP, pituitary adenylate cyclase-activating polypeptide-38.

of the kinetics of CA release upon a single fusion event as well as the estimation of net granule content (Schroeder et al., 1996).

The cellular mechanisms implicated in the regulation of the quantal size are currently receiving considerable attention (fore review, see Sulzer and Pothos, 2000). Both CA uptake into secretory vesicles and their intragranular complexation have been implicated in the regulation of vesicular volume and quantal size (Colliver et al., 2000a). On the other hand, the kinetics of exocytosis would depend on fusion pore dynamics and the granule matrix expansion, two cellular processes that are interrelated (Amatore et al., 2000).

Although the effects of cAMP on secretion have been widely studied, in this article, we present the first work on the effects of cAMP on exocytotic single events. We show that cAMP, acting on PKA, modifies the CA content of secretory events and modulates the kinetics of the last step of exocytosis. Because of the similarities of chromaffin granules and large dense cored vesicles of sympathetic nerve terminals, it is plausible that our results could be extrapolated to these structures, and intracellular cAMP levels could be modulating the synaptic performance.

Experimental Procedures

Materials. C-PTIO was acquired from Sigma/RBI (Natick, MA), H-89 from Biomol (Plymouth Meeting, PA), and pertussis toxin from Invitrogen (Barcelona, Spain). Urografin was obtained from Schering España (Madrid, Spain). Culture plates were from Corning Costar (Cambridge, MA). All other drugs, culture media, and sera were purchased from Sigma-Aldrich (Madrid, Spain). All salts used for buffer preparation were reagent grade.

Culture of Chromaffin Cells. Bovine adrenal chromaffin cells enriched in adrenaline were prepared as described elsewhere (Moro et al., 1990). Cells were plated on 12-mm diameter glass coverslips at an approximate density of 5×10^5 cells/coverslip in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum containing 50 IU/ml G-penicillin and 40 μg/ml gentamicin. Cells were maintained at 37°C in a 5% CO_2 environment and used at room temperature between 1 and 4 days of culture.

Amperometric Detection of Exocytosis. Carbon fiber microelectrodes were prepared as described by Kawagoe et al. (1993). Carbon fibers with a 5-μm radius (Thornel P-55; Amoco Corp., Greenville SC) were the kind gift of Prof. R. M. Wightman (University of North Carolina, Chapel Hill, NC). Electrochemical recordings were performed using an Axopatch 200B (Axon Instruments, Foster City, CA) (for details, see Machado et al., 2000).

Cells were washed in Krebs-HEPES buffer solution containing 140 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 2 mM $CaCl_2$, 11 mM glucose, and 10 mM HEPES, at pH 7.35 and placed in a perfusion chamber positioned on the stage of an inverted microscope (DM-IRB; Leica, Wetzlar, Germany). Amperometric measurements were performed with the carbon fiber microelectrode gently touching the cell membrane. Cell release was stimulated by 5-s pressure ejection of 5 mM Ba^{2+} from a micropipette placed 40 μm away from the cell. Barium does not require receptor activation or membrane depolarization, and it produces a low frequency of secretory events, called "spikes"; during spike analysis, the initial and final points of each event can be easily distinguished.

Data Analysis. Amperometric signals were low-pass filtered at 1 KHz, sampled at 4 KHz, and collected with locally written software using a commercial G programming language (LabVIEW for Macintosh, National Instruments, Austin, TX). The analysis of an individual exocytotic event was done through the measurement of the following parameters: I_{max}, maximum oxidation current; $t_{1/2}$, spike width at half height; Q, spike net charge; m, ascending slope of spike; and tP, time to reach the spike maximum (see figure inserted in Table 1 and Machado et al., 2000 for details). Data analysis was carried out using locally written macros for IGOR (WaveMetrics, Lake Oswego, OR) (Segura et al., 2000). These macros and their user manual can be downloaded for free from URL: http://webpages.ull.es/users/rborges/

To overcome the day-to-day variations in electrode sensitivity and cell responsiveness (Colliver et al., 2000a), effects of drugs on secretory spikes were alternated with control experiments carried out under the same conditions. Statistical analysis was performed by the nonparametric Mann-Whitney rank sum or Kolmogorov-Smirnov tests.

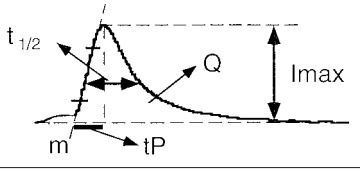
cAMP Measurements. Cells were cultured on 24-well plates at 5×10^5/well for 48 h. Cells were preincubated in Krebs-HEPES buffer containing 500 μM 3-isobutyl-1-methylxanthine (IBMX) for 15 min. Drug testing was assessed by incubating the cells for another 15 min in the presence of IBMX. Cyclic AMP measurements were done with the cAMP enzyme immunoassay kit (RPN225; Amersham-Pharmacia Biotech, Cerdanyola, Spain). Data are expressed in picomoles per microgram of total protein; proteins were measured by the bicinchoninic acid method, following the instructions given by the manufacturer (Sigma-Aldrich).

HPLC Analysis of CA. Twenty-four hour old chromaffin cells at 100,000 cells per well were washed twice in saline and incubated with saline (control) or 500 μM IBMX for 15 min. Then, half of the IBMX-treated wells were challenged with 1 μM isoprenaline for 40 s. The stimulation was stopped by adding ice-cold lysis buffer containing perchloric acid (0.05 N), Triton X-100 (0.25%), and the internal standard dihydroxybenzyl amine (100 nM). Cells were detached mechanically from the bottom, centrifuged for 3 min at 10,000*g*, and injected into an HPLC with an electrochemical detection as described previously (Borges et al., 1986).

TABLE 1

The effects of forskolin on secretory spike parameters

Cells treated with 100 nM forskolin are compared with their own control group (see results). Secretion is elicited by 5 s application of 5 mM $BaCl_2$. All values obtained from forskolin-treated cells are statistically different ($p < 0.001$) from controls (Mann-Whitney and Kolmogorov-Smirnov[a] tests).



	I_{max}	$t_{1/2}$	Q	m	tP[a]	n cells	n spike s
	pA	*ms*	*pC*	*nA/s*	*ms*		
Control	72.6 ± 2.9	16.0 ± 0.5	1.4 ± 0.06	24.2 ± 1.1	18.2 ± 1.0	12	778
Forskolin 100 nM	64.3 ± 4.6	32.9 ± 1.5	2.1 ± 0.13	17.5 ± 1.6	26.1 ± 1.9	7	308

Results

Forskolin Produces a Rapid Increase in Granule Content. The secretagogue used in our experiments, 5 mM $BaCl_2$ applied for 5 s, usually evokes exocytosis at a low rate (2.8 ± 0.3 spikes/s). The number of exocytotic events is not modified by the incubation with 100 nM FSK for 10 min (3.1 ± 0.4 spikes/s), although the total amount of CA secreted is increased. The coincubation with the nonspecific phosphodiesterase inhibitor IBMX results in a further increase in the total CA release (Fig. 1). These data suggest that the increase of CA secretion is caused by an increase in the granular content of amines. Tables 1 and 2 show that the Q increases by 50%. The effect of FSK on granule charge occurs within 2 to 5 min and is potentiated by 500 μM IBMX. Given alone, higher IBMX concentrations (5 mM) do not cause "per se" effects on spike charge or produce further increase in Q (Table 2).

Forskolin Slows and Alters the Exocytotic Process. Fig. 2a shows histograms of $t_{1/2}$ from spikes obtained after 10-min incubation with 100 nM FSK. Fig. 2b shows how FSK affects the time course of individual exocytotic events; these representative spikes are plotted using the main spike characteristics taken from Table 1. Normalized data are shown on Table 2. The effects of 100 nM FSK are also observed with higher concentrations (1 and 10 μM, data not shown), although it does not follow a clear concentration-dependence, probably because of the lack of specificity.

The spike shape changes dramatically after treatment with FSK. To characterize the kinetic parameters of spikes, we usually consider only those spikes that satisfied some requirements. Hence, we have only included in the study spikes with an I_{max} range from 4 to 500 pA or $t_{1/2}$ from 3.5 to 250 ms. We also rule out spikes with a Q over 7 pC, a time course altered by a giant prespike feature or distorted shapes by a superimposed spike. However, isolated spikes with a nonstandard shape occasionally occur. These "abnormal spikes" account for less than 3% in untreated cells, but their number increase up to 44% upon a treatment with 100 nM FSK. Some examples are illustrated in Fig. 3. Although neither kinetic parameter measurements nor mathematical models can yet precisely describe these abnormal spikes, all of them exhibit a long $t_{1/2}$, a short I_{max}, and a large Q. Besides this, they usually have long tP (Tables 1 and 2), large time decaying values and multistep ascending slopes. The number of "unshaped" spikes and the extent of their alterations increase upon the coincubation with IBMX.

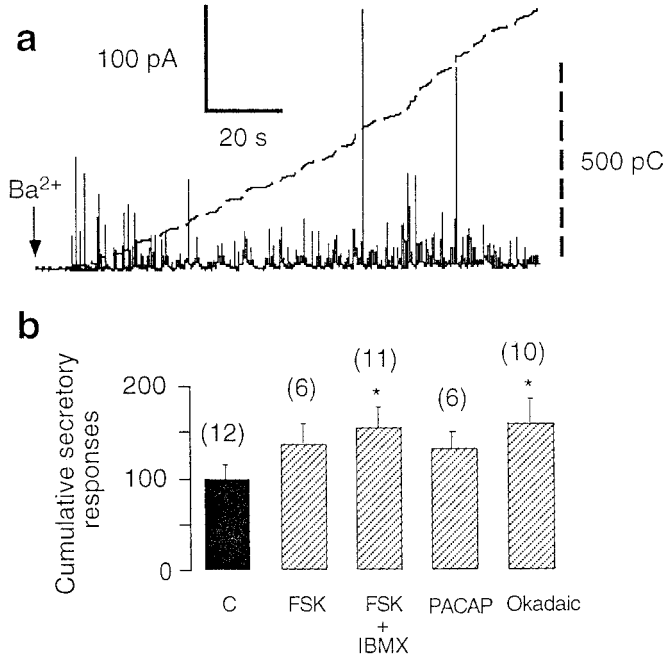


Fig. 1. Effects of forskolin on Ba^{2+}-induced secretory responses of individual bovine chromaffin cells. a, 5 mM Ba^{2+} pulse is applied for 5 s (arrow). Figure shows an original amperometric trace and the cumulative secretion obtained in the presence of 100 nM FSK (dashed trace). Calibration for time and amperometric current are indicated with solid bars; dashed vertical bar is the calibration for the cumulative secretion, expressed in picoCoulombs. b, pooled data. Bars show normalized secretion pooled from different cells (numbers in brackets) and obtained in the absence (C) or in the presence of 100 nM FSK; FSK on cells incubated with 500 μM IBMX, 10 nM PACAP, or 30 nM okadaic acid (means ± S.E.M.). *$p < 0.05$ by Student's t test.

Cyclic AMP Analogs Mimic the Effects of Forskolin Treatment. Incubation of the cells for 30 min with the cell permeant cAMP analog dB-cAMP (300 μM) results in a slowing of the degranulation speed without any effect on granule CA content. Table 2 shows the kinetic parameters from secretory spikes obtained under dB-cAMP treatment compared with their own control cells. The effects of dB-cAMP are time-dependent and are not observed along the first 30 to 60 s of drug incubation.

Isoprenaline and PACAP Reproduce the Effects of Forskolin. Isoprenaline, a non selective β-receptor agonist, and PACAP are substances that produce, besides other effects, a receptor-dependent adenylate cyclase activation. They are used to produce rapid changes in cAMP. Isoprenaline is applied either by incubation (10 μM for 10 min before Ba^{2+} stimulus) or by 5-s pressure pulse, together with Ba^{2+} stimulus, near the cell. It assures a quick cessation of the β-receptor activation after the stimulating pulse. Isoprenaline produces a very modest increase in the intracellular cAMP level (see below), which is accompanied by changes in the spike shape that are qualitatively similar to those observed with FSK. Isoprenaline given alone does not affect the apparent net CA content; however, when it is puffed to cells incubated with IBMX, its effects on secretory spikes are potentiated and associated with an increase in the granule content of CA (Table 2).

The effects of isoprenaline on spike shape and on granule charge do not occur simultaneously. The slowing of exocytosis is evident within 15 to 30 s after the β-agonist application, whereas changes in the apparent net charge (Q) are appreciated 20 to 30 s later (Fig. 4). In addition, the increase in Q reverts before the enlargement of $t_{1/2}$, probably indicating a different sensitivity to cAMP levels.

The effects of PACAP on secretory spikes are shown in Table 2. This agent produces a rapid slowing in the exocytosis kinetic, which is accompanied by a significant increase in the apparent content of CA of secretory events.

Newly Synthesized CA Do Not Explain the Increase in Granule Content. To discard the hypothesis that the observed increase in Q upon isoprenaline stimuli in IBMX-treated cells is caused by newly synthesized CA, we used HPLC to study the cell CA content and the ratios between noradrenaline, adrenaline, and dopamine. We compared control cells with IBMX- and IBMX + isoprenaline-treated cells. We assumed that a sudden activation of tyrosine hydroxylase

would produce an increase in the proportion of dopamine and that noradrenaline would increase over adrenaline, but to a lesser extent. However, the dopamine/adrenaline ratio changes only from 0.05 to 0.25%, and the total cell content of CA increases nonsignificantly by 1.3 ± 0.7%, which is not enough to justify the 68% increase in Q observed after 60 s of isoprenaline stimulation (Fig. 4).

Forskolin Partially Reverts the Effects of NO Withdrawal. We have recently reported that NO, present within chromaffin cell culture produces a drastic slowing of exocytosis through the cGMP/PKG pathway, which is apparent upon NO withdrawal (Machado et al., 2000). Carboxy-PTIO is considered to be a specific NO scavenger. To study the role of PKA on conditions of reduced PKG activity, we have examined the effects of FSK on spike shape in cells treated with C-PTIO. Cell incubation with 10 nM C-PTIO results in a drastic acceleration of exocytosis, because the I_{max} and m values of the spikes increase, whereas $t_{1/2}$ and tP decrease. However, no changes in CA content of secretory events are observed (Table 2). The addition of 100 nM FSK partially reverts this effect but also increases the granule content. The possible origin of rapid changes of net CA content within granules will be discussed below. The effects of treatment with C-PTIO in the absence or presence of FSK are shown in Fig. 5.

Intracellular cAMP as Regulator of Exocytosis. Cholera toxin, a substance that stimulates G_s proteins/adenylate cyclase, increasing the levels of cAMP, mimics the effects of FSK (Table 2). To a lesser extent, changes in spike kinetics are observed upon incubation with pertussis toxin, which blocks the α subunit of G_i proteins. Conversely, rolipram, a drug that specifically blocks phosphodiesterase type IV and delays cAMP catabolism, also causes a slowing of exocytosis accompanied by an increase in Q.

The signal transduction of cAMP seems to be the classical PKA route because the PKA inhibitor H-89 significantly attenuates the effects of FSK (Table 2). In addition, cell incubation with okadaic acid for 20 min at concentrations that produce inhibition of phosphatase IIa (3 nM) causes effects similar to those observed with FSK. Higher okadaic acid concentration (30 nM), which also inhibits phosphatase I, causes further increase in $t_{1/2}$ and Q (Table 2).

Exocytosis Is Affected by Low Levels of cAMP. For this study, we used moderately low concentrations of agonists that were generally applied as brief pulses. Changes in spike shape and Q occurred within 15 to 60 s (Fig. 4). It is difficult to address these effects to the real concentrations of free intracellular cAMP reached during these brief treatments. To attain measurements in the range of commercial kits, we have to apply stimulus for minutes and inhibit cAMP degradation with IBMX for 15 min. Nevertheless, isoproterenol does not cause significant cAMP increase (4.2 ± 0.3 pmol/μg of protein), whereas forskolin causes only a modest elevation at 0.1 and 1 μM, although it becomes evident over 10 μM (32.2 ± 6.1 pmol/μg of protein).

Discussion

Quantal release of neurotransmitter is far from being a simple "all or none" process in which secretory vesicles release a fixed amount of neurotransmitter. It becomes clear that the vesicular CA content can vary to a considerable extent (Colliver et al., 2000b; Sulzer and Pothos, 2000). In addition, secretory vesicles can experience total or partial fusion (Albillos et al., 1997, Alés et al., 1999). Recent reports have shown that intracellular signaling by PKG (Machado et al., 2000) or PKC (Graham et al., 2000) are able to modulate the kinetics of exocytosis. It is therefore likely that the number of putative second messengers regulating the vesicle content, type of fusion, or the kinetics of exocytosis will grow in the near future. In this article, we have analyzed the contribution of the cAMP/PKA system on the regulation of CA content within chromaffin granules and the kinetics of a single event of exocytosis.

The activation of PKA has been found to cause an increase in the secretory response of adrenomedullary glands (Alvarez et al., 1997) and cultured chromaffin cells (Morita et al., 1987; Przywara et al., 1996). The discrepancies found in the literature concerning the inhibitory role of cAMP on CA secretion (Baker et al., 1985; Cheek and Burgoyne, 1987) could be caused by the nature of the stimulus or the concentration of FSK (Gandía et al., 1997). In any case, it has been assumed that an augmented secretion means an augmented number of exocytotic events. However, a comparison of the

TABLE 2

The effects of various cAMP/PKA activators and blockers on secretory spike parameters

Data are normalized as the percentage of their own control group. Secretion is elicited by 5 s application of 5 mM $BaCl_2$. Statistical analysis (Mann-Whitney and Kolmogorov-Smirnov[a] tests) are performed on original data. Significant differences.

	I_{max}	Q	$t_{1/2}$	m	tP[a]	ns	nc
Forskolin (FSK) 100 nM	89*	150*	205*	72*	143*	308	7
FSK + IBMX 500 μM	60*	187*	281*	19*	335*	359	11
IBMX 5 mM	73*	104	128*	54*	135*	1286	16
ISO 1 μM ¥	78*	98	113*	63*	97	480	12
ISO 10 μM	63*	96	141*	44*	200*	395	9
IBMX 500 μM + ISO 1 μM ¥	65*	144*	197*	56*	129	411	14
PACAP 10 nM (puffed)	73*	111*	148*	54*	163*	890	9
Pertussis toxin (10 ng/ml)	97	106	109*	91	95	620	15
Cholera toxin 1 ng/ml	62*	120*	180*	42*	210*	947	18
dB-cAMP 300 μM	67*	108*	158	38*	149*	1138	14
Rolipram 3 μM	51*	125*	205*	20*	146*	777	15
H-89 50 nM + FSK 100 nM	110‡	62‡	67‡	166‡	79‡	1035	9
Okadaic acid 3 nM	73*	168*	195*	36*	192*	533	15
Okadaic acid 30 nM	93	203*	208*	49*	231*	922	8
C-PTIO 10 nM	156*	95	65*	181*	67*	367	13
C-PTIO + FSK 100 nM	128#	126#	107#	108#	104#	562	15

ISO¥, isoprenaline puffed for 5 s; ns, number of spikes; nc, number of cells.

* $p < 0.001$ with respect to control and # $p < 0.01$ compared with C-PTIO 10 nM; ‡ $p < 0.001$ between FSK and FSK on H-89 treated cells.

data from Fig. 1 and Table 1 indicates that the increase in the total amount of CA secreted comes mostly from a net enlargement in Q, not in the frequency of firing.

The stimulation of adenylate cyclase produces two main effects on exocytosis. Light stimuli, as from isoprenaline, pertussis toxin, or low dB-cAMP concentrations, causes only a slowing of the process, rendering spikes with large $t_{1/2}$ and tP as well as small I_{max} and m. Strong stimulation (e.g., isoprenaline + IBMX, FSK, PACAP, or cholera toxin) produces, in addition, an increase in Q (Table 2). This observation contrasts with that found with PKC or PKG activation in which changes in spike shape were not accompanied by changes in Q (Machado et al., 2000; Graham et al., 2000).

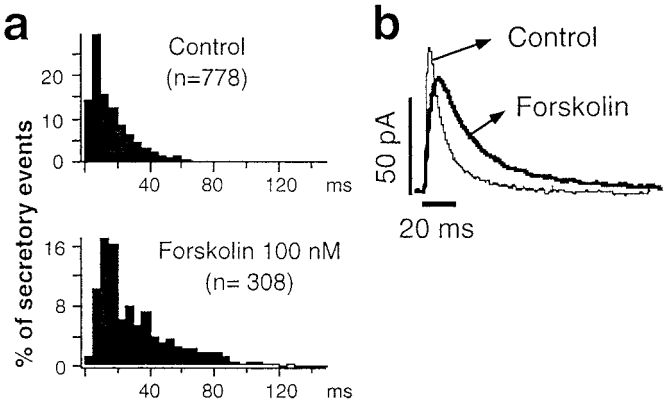


Fig. 2. Effects of forskolin on time course of exocytosis. Secretion is elicited by 5-s application of 5 mM $BaCl_2$. a, histograms of $t_{1/2}$ obtained from spikes from untreated and FSK (100 nM) treated cells for 10 min. Spikes are grouped in 5-ms bins. b, representative traces are plotted from data of Table 1. Control spike is indicated by the thin line and FSK by the thick line.

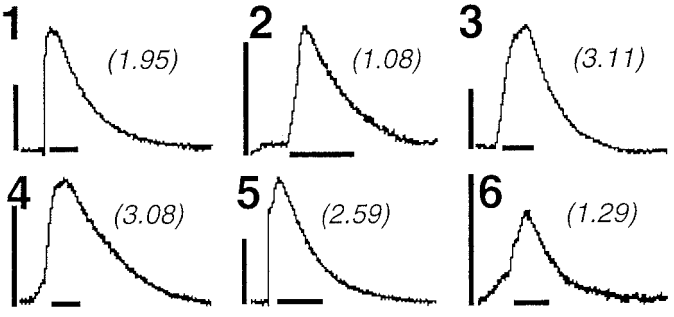


Fig. 3. Effects of forskolin on spike shape. Upon forskolin incubation, a number of spikes with atypical morphology are observed. Secretion is elicited by 5-s application of 5 mM $BaCl_2$. Some examples are shown: 1, typical well shape spike but wider and shorter with a large charge. 2, wider spike with a slow ascending and descending slopes. 3 and 4, large charge spikes with an "S" ascending slope and a long tP. 5, double ascending slopes. 6, triple ascending slopes. Calibration bars: vertical bars, 10 pA; horizontal bars, 50 ms. Numbers in brackets indicate their net charge expressed in picoCoulombs.

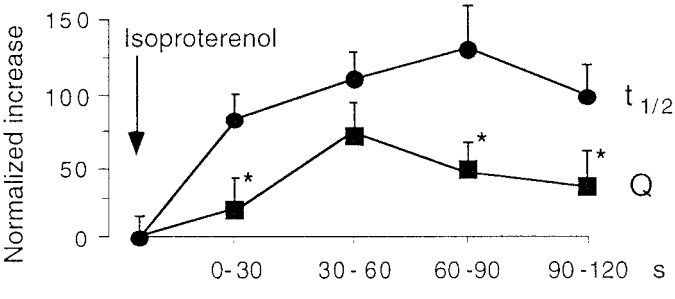


Fig. 4. The increase in the granule content of CA and the slowing of exocytosis exhibited a different time course. Cells are treated with IBMX (500 μM) for 10 min and the secretion is elicited with 5-s application of 5 mM $BaCl_2$. Secretion is recorded for 2 min and Q and $t_{1/2}$ averaged and normalized to a previous record of 30 s. Cells then receive a 5-s pulse of isoprenaline (1 μM). Time course of Q (squares) and $t_{1/2}$ (circles), upon isoprenaline application, are shown. $*p > 0.05$, indicates significant differences between both time courses as measured by Student's t test.

The stimulation with isoprenaline on IBMX-treated cells evokes changes on spike shape and Q that follow different time courses. Figure 4 shows that the slowing is observable within the first 30 s after isoprenaline application, whereas Q values increase progressively and become evident only 30 to 40 s later. Conversely, reversion of isoprenaline effects on Q precedes $t_{1/2}$. Forskolin requires more time to cause its effects, probably because it needs to permeate the cell membrane to activate adenylate cyclase.

We cannot reach a conclusive explanation for the augmentation of the vesicular amine content. In theory, it could be caused by (1) an increase in CA synthesis (Rodríguez-Pascual et al., 1999), (2) an activation of VMAT (Nakanishi et al., 1995), (3) a decrease in the CA gradient toward the vesicle (Schroeder et al., 1996), or (4) a result of compound fusion (Alvarez de Toledo and Fernández, 1990, Cochilla et al., 2000). An increase in the rate of CA synthesis should not account for the rapid changes observed in vesicular content nor explain what occurs with kinetics of exocytosis. A newly synthesized dopamine molecule has to cross the granule membrane once to be converted in noradrenaline and thrice to be transformed to adrenaline. In addition, quantification of amine content by HPLC yields a significant increase in neither total CA nor the dopamine/adrenaline ratio to support the contribution of new synthesis to the granule content within 30 to 40 s (Fig. 4). Changes in granule content of CA by reserpine or levodopa treatment (Colliver et al., 2000b) or dopamine D_2 receptor activation (Pothos et al., 1998) require tens of minutes.

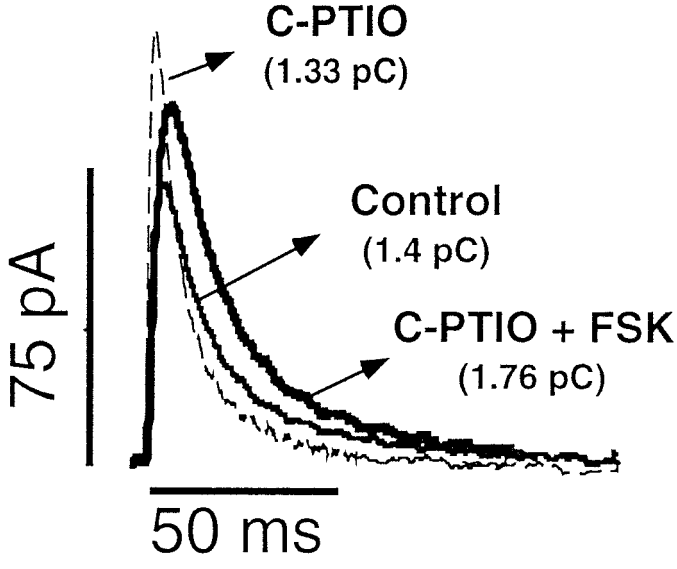


Fig. 5. The effect of forskolin in the absence of NO. Spikes are made by averaging absolute data from Table 2. Cells are stimulated by pressure injection of 5 mM $BaCl_2$ for 5 s. Untreated cells (control) are compared with cells incubated for 10 min with C-PTIO (10 nM) or C-PTIO plus FSK (100 nM). Charge values are expressed in brackets. Solid vertical and horizontal lines indicate calibration of oxidation current and time, respectively.

The granule uptake of CA is carried out by the VMAT, a H^+/monoamine antiporter, coupled to the V-type ATP-dependent H^+-pump (Henry et al., 1994). This pump creates a proton gradient that maintains an intragranular pH of around 5.5 that corresponds to the isoelectric point of chromogranin A (Blaschko et al., 1967; Yoo and Albanesi, 1990). Chromogranin A is the major granule matrix component that has been considered to play an important role in the intragranular complexation of soluble products (Helle et al., 1985; Borges et al., 2000). Acidification of the intragranular environment could increase the affinity of chromogranin A for CA, thereby reducing the free CA present within the granule. Therefore it would decrease the transmembrane gradient and favor its intragranular accumulation. This effect on the chromaffin granule matrix would also explain the dramatic slowing of the exocytosis observed. However, CA transport driven either by direct VMAT activation or through the H^+-pump/pH-gradient are too slow to account for the observed increase in the granule content within 30 to 40 s. The estimated turnover of VMAT is about 2 molecules/s, assuming 20 VMAT molecules/vesicle (Gasnier et al., 1987), this would mean an influx of 40 molecules/s, which for 40 s would equal 1600 molecules of CA/vesicle. Considering that the content of a granule is about 1 to 5 $\times$ 10^6 molecules (Winkler and Westhead, 1980), these mechanisms would then require several hours to produce a significant increase in the net CA content.

It is also possible that, under control conditions, granules do not totally release their contents and that PKA activation would promote complete emptying. Although amperometry cannot detect the CA not released, the profound slowing of the exocytotic processes caused by cAMP is not compatible with a mechanism that forces the complete emptying of chromaffin granules.

We propose that the increase in Q could be the result of compound fusion (i.e., two or more granules that fuse before exocytosis). This also could explain the changes observed in spike shape (Fig. 3). These distortions are also observed on isolated spikes, suggesting that they do not come from coincident events although they could have originated from a "double granule". Amperometry cannot conclusively address the changes observed in spike shape to a compound fusion. However, mathematical deconvolution of secretory spikes is interpreted to indicate that they could have originated from a complex phenomenon. Such a complex phenomenon could include a granule with multiple intravesicular matrices that expand at two or three different kinetics (Sánchez et al., 1999).

Histograms of spike charge resulting from PKA activation do not seem to reveal the presence of two populations of vesicle size (Fig. 6a). However, mathematical simulation helps to reveal the underlying phenomenon resulting from PKA activation. When the charge of all of the spikes is increased by 50%, similar to the average elevation caused by 100 nM FSK, the histogram is displaced to the right without changes to its shape. However, when 30% of spikes are randomly combined, the histogram reproduces what occurs when strong stimulation of adenylate cyclase produces high cAMP levels (Fig. 6b). The analysis of histograms reveals a skew to the right, which is compatible with the exocytosis of 30 to 45% of perfused granules. Granule-to-granule fusion has been shown recently to be forced by cAMP in rat pituitary lactotrophs (Cochilla et al., 2000).

Our results also clearly show that all of the cell treatments described to increase cAMP synthesis (isoprenaline, PACAP, FSK) or to inhibit its degradation (IBMX, rolipram) promote the slowing of exocytosis (Fig. 2 and Table 2). Although not conclusive, the effects observed on secretory spikes can be produced from granules with highly compacted and stored CA and not by fusion pore flickering, provided that granules, at that stage, must be almost completely fused (Schroeder et al., 1996; Amatore et al., 2000).

The cellular route used by cAMP to produce its effects seems to be the stimulation of PKA, because H-89 partially antagonizes the effects of FSK. In addition, the inhibition of protein phosphatases IIa and I with okadaic acid has the opposite effects. However, the participation of other second messengers cannot be overruled. For instance, rising cAMP stimulates Ca^{2+} entry, which also has been described as causing the aggregation of granules (Caohuy et al., 1996). Increased calcium can itself promote the presence of giant spikes (Jankowski et al., 1992). The importance of protein dephosphorylation in granule-to-granule fusion is revealed by okadaic acid treatment, which doubles the CA content and distorts the spike shape, increasing the tP over 230% (Table 2). The transduction mechanisms employed by PKA to cause

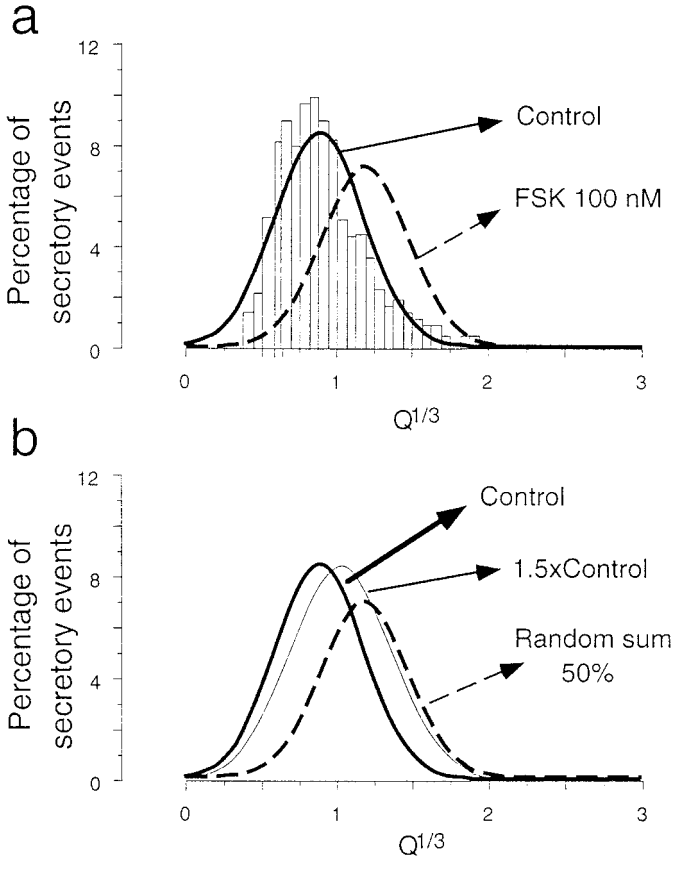


Fig. 6. Simulation of compound exocytosis explains the changes observed in granule charge. a, histograms of $Q^{1/3}$ from control cells and from cells treated with 100 nM forskolin (dashed line). Secretion is elicited by 5-s application of 5 mM $BaCl_2$. Trace lines are the computer fits to a Gaussian function. b, Gaussian fits of histograms from untreated cells (thick solid line), simulation resulted from a 50% increase of control values of Q (thin solid line) and from a random combination of 30% of spikes from the control population.

the slowing of exocytosis are still mysterious. We propose that conformational changes in chromogranin A would increase its affinity for CA, modifying the emptying of granules (Borges et al., 2000).

We recently described the presence of appreciable NO amounts surrounding cultured chromaffin cells. This NO tone keeps PKG in a basal activation state that is evidenced upon withdrawal of NO with specific scavengers such as C-PTIO (Machado et al., 2000). Experiments from Fig. 5 are done to check whether an elevation on cAMP still affected both kinetics of exocytosis and granule charge under conditions of NO deprivation. NO basically affects the kinetics of exocytosis, whereas the addition of FSK increases the net content of CA. Figure 5 and Table 2 show that spikes with higher I_{max} than control also result in a drastic increase of Q. These experiments suggest, although do not prove, that both PKA and PKG act independently, modulating the late step of exocytosis.

The experiments presented in this article show that cAMP, probably acting on PKA, modulates the kinetics of exocytosis and increases the quantal size of secretory vesicles. A carbon fiber electrode gently touching a cell membrane probably "sees" the released CA as a postjunctional cell and the noradrenaline released by a sympathetic nerve terminal. Therefore, a reduction in the I_{max} will mean a lower concentration of CA reaching the postsynaptic cell surface and a large Q, a net increase in the total CA, released by a single fusion phenomenon. The results presented here could indicate that transient variations in the level of presynaptic cAMP would be capable of producing rapid and reversible modifications in the synaptic performance.

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