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Novel synthetic sulfoglycolipid IG20 facilitates exocytosis in chromaffin cells through the regulation of sodium channels

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Running title: *Sulfoglycolipid IG20 facilitates exocytosis in chromaffin cells*

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LIST OF ABBREVIATIONS

BCC	Bovine Chromaffin Cell
BK	Big conductance K^+ channels
DMEM	Dulbecco's Modified Eagle's Medium
ER	Endoplasmic reticulum
Fura-2AM	Fura-2 acetoxymethyl ester
S1P	Sphingosine-1-phosphate
SERCA	Sarco-endoplasmic Reticulum Calcium ATPase
TG	Thapsigargin
TTX	Tetrodotoxin
VACC	Voltage-activated Calcium Channels

ABSTRACT

In searching for druggable synthetic lipids as potential modulators of synaptic transmission and plasticity, we synthesized sulfoglycolipid IG20 that stimulates neuritic outgrowth. Here we have explored its effects on ion channels and exocytosis in bovine chromaffin cells (BCCs). IG20 augmented the rate of basal catecholamine release. Such effect did not depend on Ca^{2+} mobilization from intracellular stores; rather, IG20-elicited secretion entirely dependent on Ca^{2+} entry through L-subtype voltage-activated Ca^{2+} channels. Those channels were recruited by cell depolarization mediated by IG20 likely through its ability to enhance the recruitment of Na^{+} channels at more hyperpolarizing potentials. Confocal imaging with fluorescent derivative IG20-NBD revealed its rapid incorporation and confinement into the plasmalemma, supporting the idea that IG20 effects are exerted through a plasmalemmal-delimited mechanism. Thus, synthetic IG20 seems to mimic several physiological effects of endogenous lipids such as regulation of ion channels, Ca^{2+} signaling, and exocytosis. Therefore, sulfoglycolipid IG20 may become a pharmacological tool to investigate the role of the lipid environment on neuronal excitability, ion channels, neurotransmitter release, synaptic efficacy, and neuronal plasticity. It may also inspire the synthesis of druggable sulfoglycolipids aimed at increasing synaptic plasticity and efficacy in neurodegenerative diseases and traumatic brain – spinal cord injury.

Keywords: Compound IG20; exocytosis; sodium channel; calcium channel; sulfoglycolipid; chromaffin cell

INTRODUCTION

It is known that cholesterol, sphingolipids, and phospholipids with saturated acyl tails coalesce to form tightly packed aggregates known as lipid rafts (Simons & Ikonen 1997). The only morphologically identifiable raft-like domain is the caveola. Association with the protein caveolin causes the cholesterol- and sphingolipid-enriched regions of the membrane to bulge into the cell forming flask-shaped pits (Razani *et al.* 2002). Interaction of ion channel proteins with caveolin appears to regulate channel function either directly by altering the channel kinetics or indirectly by affecting trafficking and surface expression (Jiao *et al.* 2008, Lee *et al.* 2009). Raft-enriched lipids such as cholesterol and sphingolipids can also exert effects on channel activity either through direct protein-lipid interactions (Epshtein *et al.* 2009) or by influencing the physical characteristics of the bilayer (Andersen & Koeppe 2007). The ability of ion channels to associate with specific lipid domains is thus likely to be an important regulatory aspect of channel physiology (Dart 2010).

One such physiological process is the membrane fusion (exocytosis) and fission (endocytosis) mechanisms underlying the Ca^{2+}-dependent release of neurotransmitters and hormones (Ceccarelli & Hurlbut 1980, Henkel & Almers 1996). These processes are regulated by sphingosine and ceramide. Thus, ceramidase has been shown to regulate the trafficking and exocytosis of synaptic vesicles (Rohrbough *et al.* 2004) and sphingomielinase modulates dopamine release from mesencephalic neurons (Blochl & Sirrenberg 1996) and PC12 cells (Jeon *et al.* 2005) as well as glutamate release from developing cerebellar neurons (Numakawa *et al.* 2003). Furthermore, sphingosine-1-phosphate (S1P) is involved in glutamate release from hippocampal neurons (Kajimoto *et al.* 2007) and facilitates spontaneous transmitter release at the frog neuromuscular junction (Brailoiu *et al.* 2002). Also, sphingosine activates synaptic vesicle exocytosis (Darios *et al.* 2009) and enhances exocytosis and endocytosis in bovine chromaffin cells (BCCs) (Rosa *et al.* 2010).

Glycolipids are sugar-attached lipids that are predominantly located in the outer plasmalemma layer. Their sulfation introduces negative charges that are determinants for interactions with proteic receptors and ion channels. They contribute to the regulation of myriad physiological functions such as cell differentiation, development, immune responses, cell adhesion, or blood clotting (Honke 2013) as well as to ion channel regulation (Dart 2010). Additionally, the sulfoglycolipid sulfatide plays a critical role in the regulation of oligodendrocytes differentiation and the maintenance of myelin and axonal structure (Marcus *et al.* 2006). It also contributes to the stabilization of the axoglial junction between the myelin loop and the axolemma at the paranode region (Girault & Peles 2002) as well as to the location of Na^+ and K^+ channels in this region (Ishibashi *et al.* 2002). Altered sphingolipid metabolism has been found in some sphingolipidoses, diabetes, atherosclerosis, and cancer (Delgado *et al.* 2007). Furthermore, changes in brain levels of sulfatides have been recently reported to occur in patients with early Alzheimer's disease (Han *et al.* 2002). These findings have been the starting point for an increased interest in drug development to interfere with sphingolipid metabolism to treat those

diseases. In this context, during the last years our laboratory has been engaged in the synthesis of various families of glycolipids and sulfoglycolipids that exhibit antimitotic activity in melanoma and glioma cells (Garcia-Alvarez *et al.* 2007, Doncel-Perez *et al.* 2013).

Recently, we synthesized a new family of sulfoglycolipids that promote neuritic growth and myelination and additionally, they act as inhibitors of astroglia and microglia proliferation (Nieto-Sampedro *et al.* 2012). The lead of this family is compound IG20, a sulfoglycolipid with a molecular structure that reminds that of endogenous sulfatide (Fig. 1). The present work was planned to test the hypothesis that IG20 could regulate the exocytotic release of neurotransmitters, as done by its parent endogenous lipidic compounds described above. We found that indeed, IG20 enhanced the release of catecholamines from BCCs through an exocytotic mechanism that depended on extracellular Ca^{2+}. This response was due to cell depolarization and the firing of action potentials that augmented cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_c$) and the exocytotic release of catecholamine. Theses effects seemed to be plasmalemmal-delimited because fluorescent IG20 was markedly confined to the cell membrane.

EXPERIMENTAL PROCEDURES

Isolation and culture of bovine chromaffin cells

All experiments were carried out in accordance with the guidelines established by the National Council on Animal Care and were approved by the local Animal Care Committee of the Universidad Autónoma de Madrid in accordance with the code of ethics and guidelines established by European Community Directive (2010/63/EU) and Spanish legislation (RD53/2013). Adrenal glands were obtained from a local slaughterhouse under the supervision of the local veterinary service. Chromaffin cells were isolated as decribed (Moro *et al.* 1990). Cells were suspended in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum, 50 IU/mL penicillin and 50 µg/mL streptomycin. For catecholamine release measurements from cell populations, cells were plated on 5-cm diameter Petri dishes at $5x10^6$ cells per dish. For studies of patch-clamp, changes in $[Ca^{2+}]_c$, amperometry, and fluorescent IG20, cells were plated on 1-cm-diameter glass coverslips (Labbox, Barcelona, Spain) at low density (5×10^4 cells per coverslip). Cultures were maintained in an incubator at 37 °C in a water-saturated atmosphere with 5% CO_2. Cells were used 1–4 days after plating. All experiments in this study were performed at room temperature (24 ± 2°C).

Online amperometric recording of quantal catecholamine release at the single-cell level

Carbon fibre microelectrodes were built and calibrated as previously described (Machado *et al.* 2008). The potentiostat was homemade (UAM workshop, Madrid, Spain) and was connected to an interface (PowerLab/4SP, ADInstruments, Oxford, UK) that digitised the signal at 10 kHz sending it to a personal computer that displayed it within the Pulse v8.74 software (HEKA Elektronik). A 700 mV

potential was applied to the electrode with respect to an AgCl ground electrode. The coverslips were mounted in a chamber on a Nikon Diaphot inverted microscope used to localise the target cell, which was continuously perifused by means of a five-way perifusion system with a common outlet driven by electrically controlled valves, with a Tyrode solution composed of (in mM) 137 NaCl, 1 $MgCl_2$, 5 KCl, 2 $CaCl_2$, 10 HEPES and 10 glucose (pH 7.4, NaOH). The high K^+solutions were prepared by replacing equiosmolar concentrations of NaCl with KCl. At the time of experiment performance, proper amounts of drug stock solutions were freshly dissolved into the Tyrode solution. These experiments were performed on cells from 1 to 4 days after culture.

Online amperometric recording of burst catecholamine release from perifused populations of BCCs

Cells were scrapped off carefully from the bottom of the Petri dish (5×10^6 cells per dish) with a rubber policeman and centrifuged at 120xg for 10 min. The cell pellet was resuspended in 200 µl of Krebs-HEPES solution (composition in mM: 144 NaCl, 5.9 KCl, 1.2 $MgCl_2$, 2 $CaCl_2$, 11 glucose, and 10 HEPES, pH 7.3 with NaOH). Cells suspended were trapped in a microchamber and perifused at a rate of 2 ml/min. The liquid flowing from the perifusion chamber reached an electrochemical detector model VA 641 (Metrohm, Herisau, Switzerland) placed just at the outlet of the microchamber, which monitors online the amount of catecholamine secreted under the amperometric mode. This amperometric strategy permits the online recording of reproducible catecholamine release responses during long time periods of 30–60 min. Catecholamines are oxidised at +0.65 V and the oxidation current was recorded on a PC computer (Borges *et al.* 1986).

Measurement of calcium in single-cells with fura-2AM

To measure the changes in $[Ca^{2+}]_c$ BCCs were incubated in DMEM containing the calcium probe fura-2 acetoxymethyl ester (fura-2AM; 10 µM) for 1 h at 37 °C. After this incubation period, the coverslips were mounted in a chamber and cells were washed with Tyrode solution. The setup for fluorescence recordings was composed of a Leica DMI 4000 B inverted microscope (Leica Microsystems; Barcelona, Spain) equipped with an oil immersion objective (Leica 40× Plan Apo; numerical aperture 1.25). Once the cells were placed on the microscope, they were continuously perifused by means of a five-way perifusion system at 1 ml/min with a common electrically controlled valves with Tyrode solution. Fura-2AM was excited alternatively at 340 ± 10 nm and 387 ± 10 nm using a Küber CODIX xenon arc lamp (Leica). Emitted fluorescence was collected through a 540 ± 20 nm emission filter, and measured with an intensified charge coupled device camera. Fluorescence images (Hamamatsu camera controller C10600 Orca R2) were generated at 1-s intervals.

Whole-cell current recordings

All recordings were obtained under the whole-cell mode of the patch-clamp technique in its perforated-patch configuration. To reach the perforated-patch configuration we used 100 mg/ml amphotericin B. For current recordings, the external cell perifusion solution had the following composition (in mM): 137 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, 10 Hepes (pH 7.4 with NaOH). Recording started when the access resistance decreased below 30 MΩ, which usually happened within 10 min after sealing. Series resistance was compensated by 80% and monitored throughout the experiment. The external solutions were rapidly exchanged using electronically driven miniature solenoid valves (The Lee Company, Westbrook, CO) coupled to a multi-barrel concentration-clamp device with a common outlet. The flow rate was 1 ml/min and was regulated by gravity.

The intracellular solution used for the recording of inward Na^+ currents (I_{Na}) and Ca^{2+} current (I_{Ca}) contained (in mM): 10 NaCl, 100 Cs-glutamate, 20 TEA, 14 EGTA, 5 Mg-ATP, 0,3 Na-GTP, 20 HEPES (pH 7,2 with CsOH). For recordings of outward K^+ currents (I_K), the intracellular solution used contained (in mM): 120 K-glutamate, 10 NaCl, 20 HEPES, 14 EGTA, 5 Mg-ATP, and 0,3 Na-GTP (pH 7.2 with KOH). Electrophysiological data on ion currents were acquired with an EPC-9 amplifier under the control of Pulse software (HEKA Elektronik).

To monitor the resting membrane potential (Vm), recordings were made under the current-clamp mode of the patch-clamp technique. Only cells with their initial resting Vm between -50 and -80 mV were tested. We considered that the cell was healthy when a depolarization above -50 mV fire some action potentials. The pipette-filling solution was as for I_K recording.

Experiments with fluorescent IG20-NBD

We used confocal microscopy with green light (480-510 nm) to visualize cells exposed to IG20-NBD and blue light (350-461 nm) to see the nuclear probe Hoechst. Then, we first preincubated the cells with Hoechst for 5 min so we could focus the microscope and after that, we applied 10 μM IG20-NBD dissolved in Tyrode solution; at the same time we took sequential images in the Z-axis. The setup for fluorescence recordings was composed of a Leica TCS SP2 confocal microscope with a x100 objective (spatial resolution based in this objective and the scanner characteristics was estimated in 60–80 nm for 2–3 pixel separations). This system allows for *z*-axis reconstruction with theoretical *z* slice of about 0.5 mm thick and sequential mode studies in double labeling experiments.

Statistical analysis

Analysis of amperometric recordings from single BBCs was carried out using IgorPro software (version 4.0.8; Wavemetrics, OR, USA). A rule was drawn at 10 pA, and only the spikes going above the threshold amplitude were considered. Total amperometric quantal charge (calculated by integrating the amperometric current over time during the stimulus duration) and the number and area of individual amperometric spikes > 10 pA were calculated by using a macro written in IgorPro software

(Mosharov & Sulzer 2005).

Data analysis of amperometric recordings in BBC populations (peak amplitudes in nA or areas in pC) were performed using Origin Pro software (version 8.6; OriginLab Corporation, Northampton, MA, USA). Results are expressed as mean ± SEM throughout.

Data analysis of ion currents and cell excitability were performed using the GraphPad Prism (version 5.01; GraphPad Software, San Diego, CA, USA).

Student's t-test was used to determine statistical significance between means when n values followed a normal distribution. When n values were non- parametric Mann-Whitney test was used. *$P < 0.05$ was taken as the limit of significance; ** and *** show a statistical significance of $P < 0.01$ and $P < 0.001$, respectively.

Materials and chemicals

IG20 was synthesized at our laboratories as previously described (Garcia-Alvarez et al. 2007); the compound was dissolved in distilled water, and 10 mM stock solution was stored in aliquots at -20ºC. The synthesis of fluorescent IG20-NBD started with a Fisher-type glycosylation of N-trifluoro-acetyl-D-glucosamine (Wolfrom & Conigliaro 1969) with oleic alcohol followed by regioselective sulfation using SO_3-pyridine complex. Removal of trifluoroacetyl group and subsequent amidation reaction with acid (Ludolph & Waldmann 2003), bearing a C6 linker and the NBD fluorophore, lead to IG20-NBD, that was dissolved in DMSO and stored at the concentration of 10 mM at -20ºC. DMEM and nifedipine were purchased from SIGMA-Aldrich (Madrid, Spain). The calcium binding probe fura-2AM was purchased from Invitrogen (Eugene, OR, USA). Thapsigargin was purchased from Abcam Biochemicals® (Cambridge, UK).

RESULTS

IG20 augments basal secretion and K^+-elicited secretion in populations of BCCs

BCCs (5×10^6) trapped in a microchamber and perifused at 2 ml/min with a saline solution containing 2 mM Ca^{2+}, revealed a stable rate of basal catecholamine release at around 10-20 nA. When challenged with a $35K^+$- solution (saline containing 35 mM K^+, low Na^+, 2 mM Ca^{2+}) given for 10 s every 3 min, cells responded with a transient surge of catecholamines that depending on the cell batch and culture, varied between 200 and 600 nA spike amplitudes. However, within the same batch of cells, repeated $35K^+$ pulses gave rise to quite reproducible spike responses along periods of 30-60 min. This permitted the exploration of the effects of a given IG20 concentration within the same cell batch, by giving first a set of $35K^+$ pulses (control responses) followed by another set in the presence of the compound, and a final set after its washout. At 1 μM, IG20 caused a tiny elevation of such basal secretion and a gradual increase of the $35K^+$ responses that although they gradually declined, they however remained elevated by the end of the experiment (Fig. 2A). At 3 and 10 μM, IG20 caused more pronounced elevations of basal secretion and superimposed on this, a gradual potentiation of the $35K^+$ responses was once more observed (Fig 2B,C). The instauration of these responses and their reversal were also gradual. Pooled normalized data indicated a concentration-dependent effect of IG20 in enhancing the $35K^+$ responses (Fig. 2D).

Effects on basal secretion were further explored by cell exposure to IG20 under resting conditions and between two sets of $35K^+$ pulses. Of interest was the pronounced elevation of basal secretion after a 30 s delay of adding 10 μM IG20, and the considerable enhancement of the $35K^+$ responses following its washout (Fig. 2E). Pooled results graphed in Fig. 2F show that areas of the basal secretion curves in μC were augmented by 3 and 10 μM IG20 in a concentration-dependent manner.

Next we explored whether IG20 elicited the same or distinct relative potentiation of the K^+-responses when cells were challenged with different K^+ concentrations, namely 17.7 mM ($17.7K^+$), 35 mM ($35K^+$), or 100 mM ($100K^+$). Fig. 3A,B,C shows example records of the K^+-elicited secretion transients before and during cell perifusion with 10 μM IG20. Note the elevation of basal secretion and superimposed on it, the enhanced K^+ secretory responses. Averaged data plotted in Fig. 3D show that the potentiation by 10 μM IG20 of the K^+-elicited secretion depended on its concentration, 4.7-fold at 17.7 mM K^+, 2.8-fold at 35 mM, and 1.6-fold at 100 mM.

IG20 augments the rate of basal quantal catecholamine release and potentiates the K^+-elicited secretion at the single-cell level

On-line recording of single-vesicle release of catecholamine was monitored with a carbon fibre microelectrode placed onto the surface of a single BCC and connected to an amperometer. Upon a few minutes of cell perifusion with a saline solution containing 2 mM Ca^{2+}, spontaneous spikes were rarely seen (Fig. 4A); this example cell, however, responded with a spike burst upon the application of a $35K^+$ challenge at the end of the experiment, indicating its viability. The cell of Fig 4B was initially

stimulated with a $35K^+$ pulse. IG20 (10 μM) was given 2 min before the application of the second $35K^+$ pulse; after a delay of about 30 s the cell begun to fire secretory spikes at increasing frequency that subsequently decreased. Of note was the drastic potentiation of secretion evoked by the second $35K^+$ challenge (that was applied immediately after IG20 removal) with respect the initial $35K^+$ pulse. In the cell of Fig. 4C, IG20 was applied for a short period of time (30 s) to prevent the alteration of basal secretion. When the second $35K^+$ pulse was applied on top of IG20, a notable potentiation of secretion was produced, in comparison with the first and third $35K^+$ pulses.

Cumulative basal secretion calculated as the number of spikes present in 5-s segments of the traces, is graphed in Fig. 4D; while spikes were practically absent in control cells, those exposed to 10 μM IG20 begun to fire secretory spikes after an initial delay of about 20 s, that reached a plateau of about 30 spikes after 2 min. IG20 also augmented the $35K^+$ responses from 10 to about 15 spikes (Fig. 4E).

Effects of tetrodotoxin, nifedipine, cadmium and thapsigargin on IG20 elicited secretion at the single-cell level

The removal of Ca^{2+} from the saline solution perifusing the cell of Fig. 5A during 3 min, and its subsequent perifusion for an additional 3-min period with saline solution containing 2 mM Ca^{2+}, did not generate any secretory activity. This cell was viable, as proven by the burst of spikes produced by a $35K^+$ pulse given at the end of the experiment. The cell of Fig. 5B was subjected to a similar protocol but with a major difference: it was initially perifused with a saline solution deprived of Ca^{2+} but containing 10 μM IG20. The switching of this solution to another containing 2 mM Ca^{2+} (but not IG20) immediately generated a sharp and sustained secretory response that gradually declined along the 3-min period of Ca^{2+} reintroduction. The cell of Fig. 5C was subjected to a similar protocol with a new element namely, the presence of 3 μM nifedipine in both the Ca^{2+}-free and the Ca^{2+}-containing saline solutions. Under these conditions, the simultaneous exposure to nifedipine and IG20 during the period of perifusion with Ca^{2+}-free saline, abolished the later secretory response elicited by Ca^{2+} reintroduction. Pooled data summarized in Fig. 5D indicate that the total secretion elicited by Ca^{2+} reintroduction in cells previously exposed to IG20 was abolished by 200 μM Cd^{2+} or 3 μM nifedipine, suggesting that such response was due to Ca^{2+} entry through voltage-activated calcium channels (VACCs) of the L-subtype (α_{1D}, $Ca_v1.3$).

We also tested the effects of tetrodotoxin (TTX; 1 μM) on the secretory effects of IG20 added to a saline solution containing 2 mM Ca^{2+}. Under these conditions, the control secretory effects of IG20 during 2 min of cell perifusion were 18.5±4.4 spikes and 14.6±8.4 pC of total secretion. TTX reduced by about 90% this response (Fig. 5E).

Whether the endoplasmic reticulum (ER) Ca^{2+} store contributed to the secretory effects of IG20 was explored using thapsigargin to deplete such store. Cells were perifused with a saline solution containing 2 mM Ca^{2+} and 1 μM thapsigargin for 5 min. Then IG20 was given in a solution containing Ca^{2+} but no thapsigargin (this compound is known to irreversibly block the sarco-endoplasmic

reticulum Ca^{2+} ATPase (SERCA; (Lytton *et al.* 1991)). Fig. 5F shows that total secretion elicited by IG20 in control cells (37.3±5.8 pC) was reduced to 27.1±7.1 pC in cells pretreated with thapsigargin; this reduction, however, did not reach the level of statistical significance.

Effects of IG20 on cytosolic calcium concentrations

The secretory response elicited by Ca^{2+} reintroduction in BCCs exposed to IG20 should be accompanied by an elevation of $[Ca^{2+}]_c$. This was tested in single BCCs loaded with fura-2AM. The two example cells of Fig. 6A initially responded with sharp transient elevations of $[Ca^{2+}]_c$ upon their challenging with a 5-s $35K^+$ pulse. After baseline recovery, the control cell in panel A was perifused during 6 min with $0Ca^{2+}$ saline solution while the cell in panel B was perifused with this same solution during 3 min followed by another 3-min period with Ca^{2+}-free solution containing 10 μM IG20. Then, Ca^{2+} at 2 mM was reintroduced; this evoked a tiny elevation of basal $[Ca^{2+}]_c$ in the control cell, while a pronounced elevation of $[Ca^{2+}]_c$ was produced in the cell previously exposed to IG20. Pooled results of cells subjected to this protocol, normalized as % of the initial $35K^+$ responses, are graphed in Fig. 6C. Both, the mean amplitude and area of the $[Ca^{2+}]_c$ responses were augmented 2.5- and 3-fold in the cells exposed to IG20, with respect control cells.

The possible contribution of Ca^{2+} release from the ER store was tested in a second series of experiments that included control cells and other cells treated with 1 μM thapsigargin applied during the period in between the initial $35K^+$ pulse and the final application of 10 μM IG20. These cells were perifused with saline containing 2 mM Ca^{2+} along the experiment. The cell of Fig. 6D responded with an initial sharp $[Ca^{2+}]_c$ transient upon $35K^+$ stimulation; the $[Ca^{2+}]_c$ had recovered baseline when IG20 perifusion generated an initial fast $[Ca^{2+}]_c$ elevation that later on rose more slowly to reach a peak and then slowly decline. The cell in Fig. 6E equally responded to $35K^+$ with a sharp $[Ca^{2+}]_c$ transient; its subsequent exposure to 1 μM thapsigargin produced a mild but sustained elevation of basal $[Ca^{2+}]_c$ due to ER Ca^{2+} release and ER Ca^{2+} depletion (Rasmussen *et al.* 1978). The subsequent addition of IG20 produced a $[Ca^{2+}]_c$ transient that gradually rose to a peak and then decayed. Pooled normalized data graphed in Fig. 6F shows that IG20 caused 1.5-fold higher increase of the Ca^{2+} peak amplitude and 2.5-fold higher increase of the $[Ca^{2+}]_c$ area in thapsigargin-treated cells, with respect to control cells. We also made experiments with the application first of a K^+ pulse and then after 3 min to allow baseline recovery, IG20 was applied for 1 min and another K^+ pulse was applied. Although peak $[Ca^{2+}]_c$ transients were unchanged, IG20 elicited a 1.5-fold increase of the normalized $[Ca^{2+}]_c$ area (0.99±0.1 in the control K^+ challenge and 1.49±0.13 in the K^+ challenge after IG20 treatment; n=18 cells, $P<0.05$).

Effects of IG20 on cell excitability and ion channel currents

Blockade of the secretory effects of IG20 elicited by Cd^{2+} and nifedipine suggests that Ca^{2+} entry through L-subtype of VACCs is implicated in such response. The opening of these channels requires

membrane depolarization and thus, it was predicted that IG20 had the potential to cause cell depolarization. This was tested using the perforated-patch configuration of the patch-clamp technique under the current-clamp mode.

The initial resting membrane potential (Vm) of the example BCC shown in Fig. 7A was about -80 mV. No spontaneous action potentials were observed, a property typical of BCCs (Orozco *et al.* 2006). After a delay of about 2 min, IG20 at 10 µM began to depolarize the cell to reach a plateau at about -40 mV. Superimposed on this plateau, a discharge of intermittent action potentials was produced. At an expanded scale these action potential exhibited an overshoot at around 10 mV and the typical afterhyperpolarisation. Pooled data from 9 BCCs (Fig. 7B) indicated an initial Vm of -70±8 mV; the resting Vm was reduced to -40±5 mV in the presence of IG20 ($p<0.001$). Of interest was an experiment performed in current-clamped cells showing that TTX (1 µM) did not affect the depolarizing effect of IG20 (Fig. 7B).

To inquire into the possible mechanism underlying the depolarizing effect of IG20, a study of the whole-cell currents through Na^+ channels (I_{Na}), VACCs (I_{Ca}), and K^+ channels (I_K) was performed using the perforated-patch configuration of the patch-clamp technique under the voltage-clamp mode. Fig. 7C shows two I_{Na} traces generated by test depolarizing pulses to -30 mV, applied from a holding potential of -80 mV. After 30-s of cell perifusion with 10 µM IG20, I_{Na} amplitude increased near 3-fold. The complete I-V curves for I_{Na} averaged from 18 cells are graphed in Fig. 7D. Control I_{Na} had a threshold potential at -50 mV, peaked at -10 mV, and had a reversal potential at around +70 mV. In the presence of IG20 the current amplitude increased at hyperpolarising voltages in such a manner that in the range -50 to -10 mV the I-V curve was shifted to the left by around 10 mV and the peak current was significantly enhanced by 133% at -40 mV, 89% at -30 mV, and 30% at -20 mV.

Fig. 7E shows example paired I_{Ca} traces obtained from BCCs voltage-clamped at -80 mV, using 2 mM external Ca^{2+} as charge carrier. I_{Ca} traces generated by test pulses to 0 mV were quite similar before and during cell perifusion with 10 µM IG20. Overlapping full I-V curves plotted in Fig. 7F exhibit a threshold voltage for I_{Ca} activation at -40 mV, with peak current at around -10 mV and a reversal potential at around +60 mV.

The effect of IG20 on I_K was explored in cells voltage-clamped at -80 mV and stimulated with a two-steps depolarizing pulse, first to 10 mV for 10 ms and subsequently to +120 mV for 400 ms. The first step served to stimulate Ca^{2+} entry and activate the Ca^{2+}-dependent component of I_K (mostly due to BK channels in BCCs) (Marty & Neher 1985). Thus, with this protocol (Fig. 8A) we could study within the same cell the voltage-dependent component of I_K ($I_{K(V)}$) and its Ca^{2+}-dependent component ($I_{K(Ca)}$). Fig. 8B shows two example I_K traces obtained in a BCC before (control) and during its perifusion during 1 min with 10 µM IG20; the $I_{K(Ca)}$ was enhanced by IG20 while the $I_{K(V)}$ was unaffected. The time course for the effects of IG20 on $I_{K(Ca)}$ of the cell of Fig. 8B is displayed in Fig. 8C; IG20 augmented gradually this current component to reach a plateau after a minute. Quantitative data from 12 cells are graphed in Fig. 8D. Normalised peak $I_{K(Ca)}$ was augmented by 25% in the

presence of IG20 ($p<0.001$). Paxilline (1 μM), a selective blocker of BK channels (Knaus *et al.* 1994) counteracted the potentiating effect of IG20 and further reduced $I_{K(Ca)}$ by 25% with respect to control. $I_{K(Ca)}$ was further reduced by 200 μM Cd^{2+} to 60±4% of control (Fig. 8D).

Insertion of fluorescent IG20 into the plasmalemma

Sulfoglycolipid IG20 contains a polar sugar moiety containing a negatively charged OSO_3^- group with an oleoyl lipophilic chain (Fig. 1). This chain may favour its insertion into the plasmalemma; nevertheless, the polar moiety may preclude its penetration into the cell. This was tested with the fluorescent derivative IG20-NBD. Worth of note is the fact that this compound triggered catecholamine release at the single-cell level, very much as the parent compound IG20 did (data not shown).

We first performed experiments to study the time course of plasmalemmal staining with IG20-NBD. Fig. 9A displays a temporal sequence of confocal micrographs taken from a BCC before its exposure to IG20-NBD (10 μM) and at different times of cell exposure to the compound. A tiny fluorescent labelling of the plasmalemma was apparent after 1.20 min, exhibiting a patchy distribution of IG20-NBD. This patchy accumulation of IG20-NBD was gradually growing with exposure time to the dye, to reach near confluence at 8 min. In two additional experiments done with different cell batches, cells with a clear-cut patchy ring alternated with others showing more diffuse fluorescence.

Fig. 9B shows a Z-axis sequence of confocal images taken from a BCC after 8 min exposure to 10 μM IG20-NBD. From top to bottom, images clearly show that fluorescence is located at the plasmalemma, showing a nitid ring at intermediate equatorial planes. The thick fluorescence ring observed in some regions (around 2 μm) could be due to IG20 being incorporated gradually into the cell through an endocytotic mechanism. Disperse fluorescence spots were seen at the bottom planes, probably due to the area of contact of the cell with the glass coverslip.

DISCUSSION

Central in this study was the finding that the novel synthetic sulfoglycolipid IG20 triggered the release of catecholamine from BCCs. This was true in experiments done in cell populations (Fig. 2E,F) as well as in those executed at the single-cell level (Fig. 4B). This release was surely exocytotic in nature because it was abolished in Ca^{2+}-free saline and re-established upon Ca^{2+} reintroduction (Fig. 5B).

Ca^{2+} entry was required for the IG20 secretory effects and this was achieved through the VACC pathway, as indicated by its suppression with Cd^{2+} (Fig. 5D). BCCs express L- as well as N-, and PQ-subtype of VACCs (Garcia *et al.* 2006) and thus the question arose on whether all channel subtypes contributed to the IG20 secretory effects. The fact nifedipine caused a drastic inhibition of the

response (Fig. 5C,D) indicated L channels were the preferred Ca^{2+} entry pathway triggering exocytosis from BCCs exposed to IG20. An explanation for this may rest in the fact N and PQ channels promptly inactivate upon BCCs depolarisation, while L channels do not (Villarroya *et al.* 1999).

The prompt increase of basal secretion upon addition of IG20 (Fig. 2B,C) seems apparently contradictory with the longer duration of the potentiating effects of the compound on the K^+ responses (Fig. 3B). It is also puzzling that during IG20 perifusion, the enhanced basal secretion was gradually decaying yet the potentiated K^+ response remained (Figs. 2B and 3B). This could find an explanation in the fact K^+-elicited $[Ca^{2+}]_c$ elevations are contributed not only by Ca^{2+} entry through VACCs but also by Ca^{2+}-induced Ca^{2+} release in BCCs (Alonso *et al.* 1999). This additional factor may not be present in the case of IG20 that causes a milder $[Ca^{2+}]_c$ elevation in comparison with K^+ (Fig. 6B).

IG20 triggered secretion by itself and additionally, the compound also augmented the response elicited by depolarising K^+ solutions both in cell populations (Fig. 2D) an at the single-cell level (Fig. 4C,E). The fact such potentiation was lesser the greater the K^+ concentration (Fig. 4D), indicated that IG20 itself could be eliciting cell depolarisation, thus explaining its ability to stimulate Ca^{2+} entry through L channels. These depolarising effects with superimposed action potentials were clearly demonstrated by the current-clamp experiments summarised in Fig. 7A,B.

More complicated could be to find the mechanism underlying the depolarising action of IG20. For instance, Na^+ channel modulation in cardiac myocytes elicited by agonists of α1-adrenergic receptors or general anaesthetics such as halothane develop slowly and is mediated by intracellular messengers (Terzic *et al.* 1993, Weigt *et al.* 1998). In this study, the drastic blockade of secretion by TTX (Fig. 5D) strongly suggests that voltage-activated Na^+ channels are involved in the secretory effects of IG20. This is supported by the observation that IG20 shifted the I-V curve for I_{Na} to the left (Fig. 7D), implying that Na^+ channels were more prone to be opened at more hyperpolarising potentials. At the end, this effect could remind that of veratridine that also augments the fraction of Na^+ channel opening but through a different mechanism namely, the delay of Na^+ channel inactivation (Ota *et al.* 1973). This augments Na^+ entry into BCCs (Kilpatrick *et al.* 1981), causes cell depolarisation (Lopez *et al.* 1995), opening of VACCs, increased Ca^{2+} entry (Kilpatrick et al. 1981), $[Ca^{2+}]_c$ oscillations (Maroto *et al.* 1994, Maroto *et al.* 1996, Lopez et al. 1995, Novalbos *et al.* 1999), and augmented secretion (Kilpatrick et al. 1981, Conceicao *et al.* 1998). However, IG20 did not elicit $[Ca^{2+}]_c$ oscillations (Fig. 6) neither it caused delayed inactivation of Na^+ channels (not shown). Thus, although at the end it causes cell depolarisation, enhanced Ca^{2+} entry and secretion as veratridine do, nevertheless IG20 could have those effects through a different mechanism namely, the opening of Na^+ channels at more hyperpolarising potentials. However, this sole action may not explain the depolarising effect of IG20 because it was not affected by TTX (Fig. 7B). An alternative explanation is that in addition of shifting the fast-inactivating Nav channels expressed by CCs (Nav1.3 and Nav1.7; (Vandael *et al.* 2015a, Vandael *et al.* 2015b), IG20 may activate some non-inactivating Na^+ channel which may sustain resting Vm and thus cause cell depolarization.

Although IG20 is water soluble, its long lipidic chain could facilitate its insertion into the plasma membrane (Fig. 1). On the other hand, its polar sulphated D-glucopyranoside moiety (Fig. 1) precluded its penetration into the cell cytosol, thus remaining confined to the cell plasma membrane as illustrated by the experiments with fluorescent IG20-NBD (Fig. 9). This insertion of IG20 into the plasmalemma was time dependent, suggesting that the compound underwent a gradual accumulation (Fig. 9A). This is compatible with the secretory effects of IG20 at the single-cell level that were initiated after 30 s and developed gradually along a 2-3 min perifusion period (Fig. 4D). Also, in cell populations the potentiation of the secretory responses elicited by K^+ developed gradually (Fig. 2B). This was also the case for the gradual augmentation of I_K elicited by IG20 (Fig. 8C). Another observation compatible with the stable insertion of IG20 into the plasmalemma was the slow reversibility of its effects upon its washout for instance, on secretion at the single-cell level (Fig. 5C). This was also observed in current-clamp experiments where IG20 elicited a gradual depolarisation that reversed very slowly upon its washout (Fig. 7A).

One may consider that a sulfoglycolipid such as IG20 that is inserted into the plasmalemma, could behave as an ionophore such as A23187 or even as a detergent such as digitonin. However, the reversible augmentation of catecholamine release (Figs. 2E, 4B), the potentiation of K^+-elicited secretion (Figs. 3D, 4E), the blockade of such responses by nifedipine and cadmium (Fig 5D), the partial depolarization elicited by IG20 in current-clamped cells (Fig. 7D), all require an intact hyperpolarized plasma membrane which is incompatible with the concept of ionophore or detergent. Furthermore, the reversibility of the effects and the fact the K^+ secretory response after a period of IG20 remains intact (Fig. 5C), further strengthen our view that in BCCs IG20 does not act as an ionophore or detergent chemical.

The plasmalemmal accumulation of IG20 could modify the rearrangement of endogenous lipids to regulate, for instance, the availability of Na^+ channels and increase their opening probability at more depolarising potentials (Fig. 7D). This is in line with some observations on the regulation of lipid rearrangement of various subtypes of Na^+ channels. For instance, the stimulation of β-adrenergic receptors in the heart augments current density through Na^+ channels of the Nav1.5 subtype, as a result of an interaction with the lipid raft caveolin-3; this lipid raft protein promotes the recruitment of Nav1.5-containing caveolae to the surface membrane (Balijepalli & Kamp 2008, Dart 2010). Two additional examples are the trafficking of epithelial Na^+ channels (ENaC) to the apical membrane in lipid rafts, in cells of mouse cortical collecting ducts (Hill *et al.* 2007) and the regulation of the activity of those channels by caveolin (Lee et al. 2009). Finally, mouse ventricular myocytes form a macromolecular signalling complex with caveolin-3 and a number of signalling molecules of the β-adrenergic receptor pathway (Balijepalli *et al.* 2006, Calaghan & White 2006).

Lipid regulation of large-conductance voltage- and Ca^{2+}-activated K^+ channels (BK; $K_{Ca}1.1$) has also been documented. Thus, in smooth muscle cells of rabbit pulmonary arteries, BK channels are activated by arachidonic acid and miristic acid (Kirber *et al.* 1992). Furthermore, fatty acids and

negatively charged single-chain lipids decrease their activity (Clarke *et al.* 2003). Additionally, docosahexaenoic acid (Hoshi *et al.* 2013) and cerebrosides (Zhang *et al.* 2014) directly activate BK channels. Exogenous IG20 could mimick the effects of these endogenous lipids to enhance $I_{K(Ca)}$ in BBCs (Fig. 8). The resting Vm in mouse and rat CCs is set by K^+ channels that are not the BK. For instance, TASK1 (Inoue *et al.* 2008) and SK channels (Vandael *et al.* 2012) are proven to set the resting Vm and action potentials of CCs. Modulation of those channels by IG20 (and by endogenous lipids, as commented above), could explain the initial depolarising effects of IG20.

The scheme of Fig. 10 summarizes the proposed sequence of steps underlying the mechanism involved in the exocytotic release of catecholamine from BCCs exposed to IG20: the compound inserts into the plasma membrane through its lipophilic moiety (1) but can not enter the cytosol because of its hydrophilic sulphate sugar moiety (2); this causes membrane depolarization by as yet undefined regulation of ion channels and the recruitment of Na^+ channels at more hyperpolarising potentials (3) to elicit superimposed action potentials (4) that will cause the opening of VACCs of the L-subtype (5), augmented Ca^{2+} entry and the elevation of $[Ca^{2+}]_c$ (6); this will finally lead to the exocytotic release of catecholamine (7) as well as to the activation of BK channels (8) that may be responsible of the afterhyperpolarisation of IG20-elicited action potentials (4).

In conclusion, novel synthetic sulfoglycolipid IG20 triggers the secretion of catecholamine and potentiates the secretory response to K^+ depolarisation. It seems this effect is initiated by a lipid type modulation of as yet undefined channels that causes Na^+ channel recruitment and ensuing action potentials. Although at this stage we do not know how IG20 acts to enhance Na^+ channel recruitment, its sulfoglycolipid nature indicates it could be mimicking the modulation of Na^+ channel kinetics by lipid rearrangement at the plasma membrane upon cell stimulation. IG20 belongs to a new family of sulfoglycolipids synthesised at our laboratory that promote neuritic growth and myelination (Nieto-Sampedro et al. 2012). Thus, it is plausible that the Ca^{2+} and exocytotic signals generated by IG20 here described, could contribute to the plasticity action of this sulfoglycolipid. In fact, preliminary results indicate that IG20 exerts neuroprotection in rat hippocampal slices challenged with veratridine, glutamate, or combined olygomycin-rotenone. Therefore, by mimicking and/or causing a redistribution of endogenous plasmalemmal sulfatide, an IG20-like sulfoglycolipid drug could represent a new therapeutic strategy to improve neurotransmission. This may find potential therapeutic applications in various CNS diseases where neurotransmission is impaired i.e. Alzheimer's disease where altered endogenous sulfatide levels have been recently described at early disease stages (Han et al. 2002).

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FIGURE LEGENDS

Figure 1. Biosynthetic pathway of endogenous sulfatide (**B**) from ceramide (**A**) and molecular structure of the synthetic sulfoglycolipid IG20 (oleoyl 2-N-acetyl-2-amino-2-deoxy-6-O-(oxosulfonyl)-alpha-D-glucopyranoside)(**C**)

Figure 2. IG20 augments the rate of basal catecholamine release and potentiates the K^+-evoked release in populations of bovine chromaffin cells (BCCs). Cells trapped in a microchamber ($5x10^6$) were perifused at 2 ml/min with a Krebs-Hepes solution at room temperature. The rate of catecholamine release was amperometrically monitored online in the perifusion solution that passed through an electrochemical detector. Once baseline secretion stabilised, cells were challenged with 10-s pulses of a K^+-enriched solution (35 mM K^+, low-Na^+, 2 mM Ca^{2+}; $35K^+$), applied at 3-min intervals. The release of catecholamine is expressed in the ordinates of the corresponding panels in nA of the current generated by the oxidation of the catecholamine. IG20 was present at the concentrations and during the time lapse indicated by the horizontal bars below each trace. **D** shows pooled data normalized as % of the pre-IG20 perifusion (C, control secretion during the third K^+ pulse) in each individual batch of cells. Data are means $\pm$ SEM of the number of cell batches (n) and cultures (N) shown in parentheses. *$p<0.05$, ***$p<0.001$ with respect to control. **E**, example trace to illustrate the augmentation of secretion elicited by IG20 in the absence of $35K^+$ stimulation. **F**, total enhanced basal secretion elicited by IG20 (ordinate in μC). ***$p<0.001$ with respect to 3 mM IG20 (Student t test).

Figure 3. Greater facilitation of secretion elicited by IG20 when using lower K^+ concentrations, with respect to higher K^+ concentrations, in perifused BCC populations. Experimental protocols are as in Fig. 2C. **A,B,C** are original traces of experiments done with 17.7 mM K^+ ($17.7K^+$), 35 mM K^+ ($35K^+$), or 100 mM K^+ ($100K^+$), respectively, repeatedly applied during 10-s at 3 min intervals, as indicated by dots at the bottom of each trace. IG20 was applied during the time period indicated by the horizontal bars below each trace. D, pooled data on peak secretion normalized as 100% (control, C) of the response before adding IG20. Data are means $\pm$ SEM of the number of cell batches (n) and cultures (N) displayed in parentheses. ***$p<0.001$ with respect to control (Student t test).

Figure 4. Effects of 10 μM IG20 on the spontaneous and K^+-elicited secretory spike events monitored amperometrically at the single-cell level with a carbon fibre microelectrode. **A**, example record showing no spontaneous events during a 5-min period of cell perifusion with Tyrode containing 2 mM Ca^{2+}, and the spike burst generated by the K^+ pulse (35 mM K^+, low-Na^+, 2 mM Ca^{2+}) applied at the end of the experiment (bottom horizontal bar). **B**, example record obtained in a BCC showing the initial K^+-evoked burst of secretory spikes over baseline elevation, the increasing number of spikes elicited by IG20 after a 30-40 s delay (bottom horizontal line), and the spike burst generated by K^+ at the end of the experiment. **C**, protocol similar to the experiment of panel B, showing the enhanced $35K^+$ secretion after 30-s pre-exposure to IG20. **D**, cumulative secretion expressed as spike number counted at 5-s intervals (ordinate) versus time (abscissa), in control BCCs and cells exposed to IG20. E, pooled data on the facilitation of IG20 of quantal secretion elicited by K^+, expressed as cumulative spike number (ordinate) counted as in D. Data in D and E are means ± SEM of the number of cells (n) and cultures (N) given in parentheses. Data passed the normality test so Student's t-test was used. *p<0,05 and **p<0.01with respect to control.

Figure 5. Augmentation of basal secretion elicited by IG20 depends on Ca^{2+} entry through voltage-activated Ca^{2+} channels (VACCs). As in Fig. 4, quantal catecholamine release from single BCCs was recorded with a carbon fibre microelectrode and amperometry. **A**, cell first perifused with a nominal Ca^{2+}-free saline ($0Ca^{2+}$) followed by 2 mM Ca^{2+} reintroduction ($2Ca^{2+}$) and a final $35K^+$ pulse, as indicated by horizontal bars at the bottom. **B**, cell initially perifused with $0Ca^{2+}$ and IG20 and subsequently with $2Ca^{2+}$ in the absence of IG20 (bottom lines). **C**, cell perifused with nifedipine throughout, initially in $0Ca^{2+}$ and IG20 and then with $2Ca^{2+}$ plus nifedipine but not IG20, and a final $35K^+$ challenge in $2Ca^{2+}$ without nifedipine. **D**, pooled results of experiments with the protocols A, B and C and additionally with other experiments using Cd^{2+} instead of nifedipine. **E**, secretory effects of IG20 (10 μM) given with saline containing 2 mM Ca^{2+} in the absence (control secretion) expressed as spike number and total secretion, or in the presence of TTX (1 μM) given 1 min before and during the 2-min exposure to IG20. **F**, pooled data of experiments performed with cells pretreated with 1 mM thapsigargin in $2Ca^{2+}$ during 5 min followed by IG20 treatment at 10 mM. Total secretion (ordinates) in D and E were calculated as the summatory of spike areas during the Ca^{2+} reintroduction period. Data in D and E are means ± SEM of the number of experiments (n) and cultures (N) shown in parentheses. Data failed the normality test so Mann-Whitney test was used.*p<0.05, ***p<0.001 with respect to control (Ca^{2+} reintroduction without any treatment).

Figure 6. IG20 augments the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) in fura-2AM-loaded BCCs. These experiments were performed following protocols reminding those used to study secretion at the single-cell level (see Fig. 5). **A**, example fluorescence trace in a cell first challenged with a $35K^+$ pulse (35 mM K^+, low Na^+, 2 mM Ca^{2+}) followed by a 6-min period of Ca^{2+} deprivation ($0Ca^{2+}$) and Ca^{2+} reintroduction, as indicated by bottom horizontal bars. **B**, example trace in a cell with a protocol similar to A in which the 6 min-period in $0Ca^{2+}$ had two parts: 3 min of initial perifusion in $0Ca^{2+}$ and 3 min in $0Ca^{2+}$ containing 10 µM IG20, as indicated; the Ca^{2+} was reintroduced in the absence of IG20 ($2Ca^{2+}$). **C**, pooled averaged data on peak and area of the $[Ca^{2+}]_c$ elevations in control cells (C) and IG20-treated cells. **D** and **E**, example traces from two cells perifused with $2Ca^{2+}$, first challenged with $35K^+$ pulses and subsequently perifused with Ca^{2+}-containing saline in the absence (control) or the presence of 1 mM thapsigargin, and finally exposed to IG20, as indicated by the bottom horizontal bars. **F**, pooled results from experiments as those in D and E, on peaks and areas of the $[Ca^{2+}]_c$ elevations produced by IG20 in control cells (D) and thapsigargin-treated cells (E). Data in panels C and F were normalized as % of the initial K^+ response within each cell; they are means $\pm$ SEM of the number of cells indicated in parentheses. *p<0.05, ***p<0.001 with respect their respective control Data did not fit D'Agostino and Pearson normality test so the Mann-Whitney test was used.

Figure 7. IG20 causes cell depolarization and shifts the I-V curves for Na^+ currents (I_{Na}) to the left, but does not affect Ca^{2+} currents (I_{Ca}). These experiments were performed with the patch-clamp technique under the perforated-patch configuration. **A**, original trace on the membrane potential (Em) of a current-clamped BCC, exposed to IG20 during the time period indicated by the top horizontal bar. **B**, pooled data on the depolarizing effects of IG20 (ordinate, in mV) and the lack of effect of TTX (1 mM) on this depolarisation. **C**, original whole-cell I_{Na} current traces obtained from a BCC before (control) or 30 s after IG20 perifusion, using the voltage-clamp protocol shown on the top. **D**, pooled results of I-V curves for I_{Na} (ordinate) obtained with sequential series of test depolarising pulses given in 10 mV steps (abscissa). **E**, original I_{Ca} current obtained from a voltage-clamped cell before and 30 s after IG20 exposure, generated by applying the protocol shown on the top. **F**, pooled data on I-V I_{Ca} curves generated by sequential application of depolarising pulses to voltage-clamped cells, given at 10-mV steps (abscissa). Data in B, D, and F are means ± SEM of the number of experiments and cultures shown in parentheses. **p < 0.01 and *** P < 0.001 with respect to basal or control.

Figure 8. IG20 augments the Ca^{2+}-dependent component of the whole-cell K^+ current ($I_{K(Ca)}$), but not the voltage-dependent component ($I_{K(V)}$) in voltage-clamped BCCs. **A**, protocol used to stimulate the cells. **B**, I_K currents with their Ca^{2+}-dependent and voltage-dependent components before (control) and 2 min after exposure to IG20. **C**, time course of I_K in an example cell before (control) and in the presence of IG20. **D**, pooled results of data from BCCs exposed to IG20 alone, or in the presence of 1 μM paxilline or 100 mM Cd^{2+}. Data are means ± SEM of the number of cells and cultures indicated in parentheses.

Figure 9. Fluorescent IG20-NBD accumulates in the plasma membrane and does not penetrate into BCCs. **A**, Time course of IG20-NBD (10 μM) accumulation into the plasma membrane of a cell before (pre-application, top image) and during its application during the times shown in each fluorescence micrograph using fluorescence confocal microscopy. **B**, sequential confocal microscopy images (left to right, top to bottom) taken on the Z axis, 8 min after cell incubation with IG20-NBD. Note the nitid accumulation of fluorescence at equatorial planes.

Figure 10. Scheme summarising the proposed sequence of events leading to augmentation or exocytotic release of catecholamine from bovine chromaffin cells (BCCs). IG20 inserts into the plasma membrane through its lipophilic moiety (1) but can not enter the cytosol because of its hydrophilic sulphate sugar moiety (2); this causes an initial cell depolarization and the subsequent recruitment of Na^+ channels at more hyperpolarizing potentials (3) to elicit TTX-sensitive superimposed action potentials (4) that will cause the opening of VACCs of the L-subtype (5), augmented Ca^{2+} entry and the elevation of $[Ca^{2+}]_c$ (6); this will finally lead to the exocytotic release of catecholamine (7) as well as to the activation of BK channels (8) that may be responsible of the afterhyperpolarization of action potentials (4).