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(54) Titre : CANAUX IONIQUES GENETIQUEMENT MODIFIES A COMMANDE NUCLEOTIDIQUE CYCLIQUE ET UTILISATION

(54) Title: GENETICALLY MODIFIED CYCLIC-NUCLEOTIDE CONTROLLED ION CHANNELS AND THE USE THEREOF

(57) Abrégé/Abstract:
The invention relates to genetically modified cyclic-nucleotide controlled ion channels, where the sub-units thereof are altered in such a manner that they exhibit a higher sensitivity for cAMP and/or a higher selectivity for cAMP in relation to cGMP in comparison with the Wildtype according to SEQ ID NO 1 and 2.

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Abstract

The present invention relates to genetically modified cyclic nucleotide-gated ion channels whose subunits have been altered so as to have higher sensitivity to cAMP and/or higher selectivity for cAMP compared to cGMP in comparison with the wild type according to SEQ ID NO 1 and 2.

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Description

Genetically modified cyclic nucleotide-gated ion channels and use thereof

The present invention relates to genetically modified nucleic acids, preferably DNAs, which code for cyclic nucleotide-gated ion channels (CNG channels) and to the corresponding proteins and the use thereof.

Chemical substances such as, for example, hormones or neurotransmitters may bind as "primary messengers" (ligands) to the membrane of cells and thereby trigger a large variety of biochemical-physiological reactions inside said cells, which enable the latter to respond to their environment. This process is mediated by a large number of membrane-bound receptors to which ligands can bind specifically and directly. It is at the beginning of various, partly extremely complex transaction cascades. Receptors control and regulate via such cascades the activity of various cellular proteins (effector proteins). These effector proteins for their part can in turn regulate the concentration of intracellular messengers, the "secondary messengers". Only such secondary messengers control a multiplicity of physiological reactions such as, for example, synthesis and release of hormones and neurotransmitters, cell division and cell growth and excitation and excitability of neuronal cells. Particularly important secondary messengers include cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP) and Ca^{2+} ions.

The intracellular concentration of secondary messengers is usually regulated by signal transduction cascades in which G protein-coupled receptors in the membrane of cells (GPCRs) register an extracellular signal then activate corresponding G proteins which in turn either

stimulate or inhibit the activity of the corresponding effector proteins (Morris A.J. and Malbon C.C. (1999) Physiological regulation of G protein-linked signaling. Physiol. Rev., 79, 1373-1430). In addition, other proteins can modulate the activity of each individual component within the framework of a large variety of feedback mechanisms.

Deviations from the physiologically normal concentration of ligands or disruptions in the course of a signal transduction cascade, caused, for example, by the malfunction of a component involved therein, may cause severe diseases. Since GPCRs represent a particularly important interface between the extracellular and intracellular medium of the cell by serving as binding site or site of attack of a very large number of endogenous and exogenous chemical substances and, moreover, controlling in a large variety important physiological processes in virtually any vital organ or tissue, they are of outstanding interest for medical-pharmacological interventions. Particularly important areas of indication in this connection are disorders of the central and peripheral nervous system, of the cardiovascular system and the inner organs.

A therapeutic goal of the pharmaceutical industry is to develop pharmacological active compounds which activate (agonists), inhibit (antagonists) the target proteins or else modulate the activity thereof. This additionally requires detailed functional characterization of the appropriate target proteins. For this purpose, various methods have been developed in recent years, which differ, some of them markedly, with respect to their flexibility, but also to the meaningfulness of the results obtained therewith, and to their robustness and their effectiveness regarding speed, amount of work required and costs.

Now that the complementary DNA of a multiplicity of receptors have been cloned and said receptors can be

expressed functionally in cell systems, the studies are carried out mainly on heterologously expressed receptors. The prior art regarding the strategies and methods used and application thereof are described, for example, in articles by Hertzberg R.P. and Pope A.J. (2000) High-throughput screening: new technology for the 21st century. Curr. Opin. Chem. Biol., 4, 445-451, Howard A.D., MacAllister G., Feighner S.D., Liu Q., Nargund R.P., van der Ploeg L.H., and Patchett A.A. (2001) Orphan G-protein-coupled receptors and natural ligand discovery. Trends Pharmacol. Sci., 22, 132-140, Civelli O., Northacker H.P., Saito Y., Wang Z., Lin S.H. and Reinscheid R.K., (2001) Novel neuro-transmitters as natural ligands of orphan G-protein-coupled receptors. Trends Neurosci., 24, 230-237, and also in the references contained therein.

More than 60% of the GPCRs known regulate the intracellular concentration of the secondary messenger cAMP. Various methods exist for studying the effect of chemical substances on such GPCRs and the corresponding effector proteins.

Some of these methods are based on direct, usually radiochemical, measurements of intracellular cAMP concentration. For this purpose, for example, the cells are stimulated, biochemically disrupted after a defined period of time and the change in cAMP concentration is determined. Although these measurement methods are very sensitive, they are usually inherently slow, cost-intensive and time-consuming. It is, moreover, not possible to monitor the change in intracellular cAMP concentration in real time. Important characteristic properties such as speed and course of an activation or inhibition can be determined only by a multiplicity of additional measurements at considerable additional expense. On the other hand, said methods are advantageous in that it is possible to study the effect of active compounds not only on GPCRs but also on the

effector proteins which regulate intracellular cAMP concentration.

Another method which may be used for measuring intracellular changes in cAMP or cGMP concentration makes use of the properties of membrane-bound CNG channels. Cyclic nucleotide-gated ion channels (CNG channels) are membrane-bound proteins which have the features and properties described below (Finn J.T., Grunwald M.E. and Yau K.W. (1996) Cyclic nucleotide-gated ion channels: an extended family with diverse functions. Annu. Rev. Physio., 58, 395-426; Richards M.J. and Gordon S.E. (2000) Cooperativity and cooperation in cyclic nucleotide-gated ion channels. Biochemistry, 39, 14003-14011). CNG channels comprise (1) presumably 4 or 5 subunits (α and/or β subunits) which (2) in each case span the membrane six times and (3) possess in each case a binding site for cyclic nucleotides at the carboxy-terminal intracellular end. CNG channels are (4) activated directly and in a manner dose-dependent by cAMP or cGMP, form (5) an aqueous pore in the membrane with a conductivity which is only slightly selective for monovalent cations and are (6) likewise permeable for divalent cations such as Ca^{2+} ions, for example.

"Binding site for cyclic nucleotides" refers to that section in the CNG channel subunits to which the cyclic nucleotides cAMP and cGMP can bind in a dose-dependent manner. The amino acid sequence in this section determines to a considerable extent the sensitivity of a CNG channel for cAMP or cGMP (sensitivity).

"Conductivity" refers to the property of ion channels of enabling in a more or less selective manner ions to flow from the outside of the cell into the cell interior or to flow out of the cell interior to the outside. For this purpose, the ion channels form an opening in the membrane (aqueous pore), through which, depending on the state of activation of said channels,

ions can flow in or out, according to the concentration gradient.

It is possible in heterologous expression systems to express functional CNG channels from identical α subunits (homooligomers; Kaupp U.B., Niidome T., Tanabe T., Terada S., Bönigk W., Stühmer W., Cook N.J., Kanagawa K., Matsuo H., Hirose T., Miyata T. and Numa S. (1989) Primary structure and functional expression from complementary DNA of the rod photoreceptor cyclic GMP-gated channel. Nature, 342, 762-766), from different α subunits (heterooligomers; Bradley J., Li J., Davidson N., Lester H.A. and Zinn K. (1994) Heteromeric olfactory cyclic nucleotide-gated channels: a subunit that confers increased sensitivity to cAMP, Proc. Natl. Acad. Sci. USA, 91, 8890-8894; Liman E.R. and Buck L.B. (1994) A second subunit of the olfactory cyclic nucleotide-gated channel confers high sensitivity to cAMP. Neuron 13, 611-621), and as heterooligomers from α and β subunits (Chan T.Y., Peng Y.W., Dhallan R.S., Ahamed B., Reed R.R. and Yau K.W. (1993) A new subunit of the cyclic nucleotide-gated cation channel in retinal rods. Nature, 362, 764-767). The β subunits alone cannot form functional channels but have exclusively modulatory functions in heterooligomeric CNG channels (Chen T.Y., Peng Y.W., Dhallan R.S., Ahamed B., Reed R.R. and Yau K.W. (1993) A new subunit of the cyclic nucleotide-gated cation channel in retinal rods. Nature, 362, 764-767). When cAMP or cGMP binds to CNG channels, said channels open in a dose-dependent manner and ions flow into the cell. Activation of the CNG channels results under physiological conditions in an increased Ca^{2+} conductivity of said channels and thus causes the increase in intracellular Ca^{2+} concentration. A change in concentration of this kind can be measured using optical Ca^{2+} measurement methods. Thus, these ion channels could in principle be used as cellular cAMP sensor for studying and characterizing any receptors

and intracellular proteins which regulate intracellular cAMP concentration. This method is very rapid, effective and inexpensive in comparison with direct cAMP measurements. It allows a high throughput of tests per day and makes real-time measurements possible. This method is therefore in principle particularly suitable for pharmacological drug screening.

The documents US 6 001 581 and WO 98/58074 and Gotzes F. (1995) Dissertation. ISSN 0944-2952 describe the use as cAMP sensor of CNG channels comprising the α3 subunits from the epithelium of the nose. However, such CNG channels have several decisive disadvantages when used as cellular cAMP sensors in pharmaceutical drug screening. As little as 2 µM cGMP activates these CNG channels but only 80 µM cAMP produces half-maximum activation thereof (Dhallan R.S., Yau K.W., Schrader K.A. and Reed R.R. (1990) Primary structure and functional expression of a cyclic nucleotide-activated channel from olfactory neurons. Nature, 347, 184-187; Ludwig J., Margalit T., Eismann E., Lancet D. and Kaupp U.B. (1990) Primary structure of cAMP-gated channel from bovine olfactory epithelium. FEBS Lett. 270, 24-29). However, since intracellular cAMP concentration usually changes only by a few µM, such CNG channels are only poorly suitable as cAMP sensors, although they are suitable as cGMP sensors in principle. Moreover, even small fluctuations in intracellular cGMP concentration can interfere with the cAMP concentration measurements.

In contrast, half-maximum activation ($K_{1/2}$ value) of heterooligomeric CNG channels composed of α3, α4 and β1b subunits is already obtained at a cAMP concentration of about 4 µM, while $K_{1/2}$ for cGMP changes only insignificantly in comparison with the homooligomeric channels (Bönigk W., Bradley J., Müller F., Sesti F., Boekhoff I., Ronnett G.V., Kaupp U.B and Frings S., (1999) The native rat olfactory cyclic nucleotide-gated channel is composed

of three distinct subunits. J. Neurosci., 19, 5332-5347. CNG channels of this kind have in principle excellent suitability as cellular cAMP sensors. Disadvantageously, however, expression of such channels in heterologous cell systems requires a lot of work and time. Moreover, small fluctuations in intracellular cGMP concentration may interfere with the cAMP sensor function.

However, it is also possible to use molecular-biological methods for preparing CNG channels which comprise only α subunits but are nevertheless highly sensitive to cAMP: in 1991, a genetically modified α3 subunit of the bovine CNG channel was described, in which subunit threonine at position 537 had been replaced with a serine (T537S) (Altenhofen W., Ludwig J., Eismann E., Kraus W., Bönigk W. and Kaupp U.B. (1991) Control of ligand specificity in cyclic nucleotide-gated channel from rod photoreceptors and olfactory epithelium. Proc. Natl. Acad. Sci. USA, 88, 9868-9872). This subunit forms CNG channels whose half-maximum activation is produced by 14 µM cAMP. Threonine T537 is located in the sequence section of the α3 subunit, which is involved to a considerable extent in binding of the cyclic nucleotides. Evidently, the amino acid in this position is particularly important for the sensitivity of the CNG channels (Altenhofen W., Ludwig J., Eismann E., Kraus W., Bönigk W. and Kaupp U.B. (1991) Control of ligand specificity in cyclic nucleotide-gated channel from rod photoreceptors and olfactory epithelium. Proc. Natl. Acad. Sci. USA, 88, 9868-9872). However, said mutation, T537S, also increases the sensitivity of the channels to cGMP ($K_{1/2}$ = 0.7 µM). Such channels (T537S mutants) can be expressed heterologously with low expenditure, but they are, as cAMP sensor, even more susceptible to interference from small fluctuations in intracellular cGMP concentration than the heterooligomeric channels. Moreover, the sensitivity to cAMP is still not high

enough in order to reliably register also small fluctuations in intracellular cAMP concentration. Furthermore, mutations in the α3 subunit of the CNG channel are known which increase sensitivity to cAMP and additionally reduce sensitivity to cGMP. (Rich T.C., Tse T.E., Rohan D.G., Schaack J. and Karpen J.W. (2001) In vivo assessment of local phosphodiesterase activity using tailored cyclic nucleotide-gated channels as cAMP sensors. J. Gen. Physiol., 118; 63-78). 1.2 µM cAMP but only 12 µM cGMP produce half-maximum activation of CNG channels composed of the rat α3 subunit in which cysteine in position 460 (C460) has been replaced with tryptophan (W) and, in addition, glutamate in position 583 (E583) has been replaced with methionine (M) (C460W/E583M mutant).

It was the object of the invention to develop CNG channels as cAMP sensors whose sensitivity to cAMP or cGMP is similar to that of the C460W/E583M mutant but which are genetically modified only in one position. Such CNG channels may be used in simple and rapid cellular measuring systems efficiently and universally for pharmaceutical drug screening but also for characterizing pharmacological or potentially pharmacological target proteins.

This object is achieved by CNG channels composed of α3 subunits which have been modified in the position corresponding to threonine T537 in the bovine α3 subunit so as to have higher sensitivity to cAMP and/or higher selectivity for cAMP compared to cGMP in comparison with the wild type according to SEQ ID NO 1 and 2.

These ion channels have a sensitivity to cAMP and cGMP similar to that of the C460W/E583M mutant.

The invention moreover relates to a method for preparing said CNG channels. The invention also relates to expression vectors comprising the nucleic acids for the modified CNG channels. The invention likewise relates to cell lines which are transformed with the described expression vectors and which can express the CNG channels. Particular preference is given to cell lines capable of coexpressing heterologously either GPCRs, adenylate cyclases, phosphodiesterases or other proteins which regulate intracellular cAMP concentration together with a modified CNG channel.

The invention further relates to a method for preparing said cell lines, which comprises carrying out a transformation by means of expression vectors. According to the invention, the genes for the proteins are preferably cloned into the expression vector, followed by transformation of the cell lines.

According to the invention, preference is given to using CNG channels composed of α3 subunits. However, other subunits from bovine or other organisms are also suitable.

In the subunits used according to the invention, preference is given to replacing the amino acid corresponding to threonine at position T537 in the bovine α3 subunit with a different amino acid other than serine. Particular preference is given here to those subunits in which threonine has been replaced with methionine or valine. SEQ ID NO 3 and 4 and, respectively, SEQ ID NO 5 and 6 depict the bovine α3 subunit as an example of subunits modified in this way.

The ion channels of the invention are especially suitable as cellular cAMP sensors for measuring intracellular cAMP concentration. They are also suitable for determining the action of ligands,

agonists and antagonists on G protein-coupled receptors (GPCRs) which regulate intracellular cAMP concentration. Moreover, they may be used for determining the action of activators and inhibitors on adenylate cyclases and phosphodiesterases (effector proteins) which regulate intracellular cAMP concentration.

The invention further relates to the use of cellular measuring systems comprising nucleic acids and the corresponding proteins for determining the action of chemical substances which influence the activity of cellular components which regulate intracellular cAMP concentration directly or indirectly.

The cellular measuring systems may be used universally and flexibly as cAMP sensors for pharmaceutical drug screening and drug characterization and for characterization of pharmacologically relevant proteins. The latter include all G protein-coupled receptors, adenylate cyclases, phosphodiesterases and any other proteins involved in cAMP signal pathways.

Cellular cAMP sensors which may be prepared and used instead of the T537M mutant or the C460W/E583M mutant however, are in principle also other genetically modified CNG channels which have
(i) a similarly high or higher sensitivity to cAMP,
(ii) a similarly high or higher sensitivity to cAMP or else
(iii) a similarly high or higher sensitivity to and, in addition, a similarly high
or higher selectivity for cAMP.

CNG channels of this kind may have (1) α3 subunits of other organisms, (2) other CNG-channel subunits from bovine or other organisms, and (3) a homooligomeric or heterooligomeric composition of these subunits. These

subunits may (4) be genetically modified in each case at the position corresponding to position T537 in the α3 subunit of the bovine CNG channel. The threonine in this position may have been replaced with a methionine or a valine or else with another amino acid (with the exception of serine). Such subunits may have (5) further genetic modifications at other positions. These CNG channels may further (6) comprise chimeric subunits which have been genetically modified in the same way at said position.

"Genetically modified at the position corresponding to position T537 in the α3 subunit of the bovine CNG channel" means the following: the different subunits of CNG channels (e.g. α1, α2, α3 and α4) or identical subunits of CNG channels of different organisms, such as, for example, the bovine and rat α3 subunits, have sequences which are highly similar to one another. Nevertheless, the position of the structurally and functionally important sections in the amino acid sequence of said subunits usually differs slightly. The skilled worker, however, is able to identify the sequence sections and amino acids corresponding to one another by comparing the sequences. Threonine T537 in the binding site for cyclic nucleotides in the bovine α3 subunit, for example, corresponds to threonine T539 in the rat α3 subunit or to threonine T560 in the bovine α1 subunit.

"Further genetic modifications" means that, in addition to a modifications of the invention, an amino acid has been replaced with a different one in at least one other position or an amino acid has been deleted from or added to at least one other position.

"Chimeric subunits" means those CNG-channel subunits which are composed of at least two different subunit moieties, i.e., for example, a subunit composed of the

amino-terminal moiety of the α1 subunit and the carboxy-terminal moiety of the α3 subunit. Such chimeras can be readily prepared by a skilled worker using molecular-biological methods and are often utilized in order to combine particular properties of one protein with the properties of another protein or to transfer particular properties to another protein or else to alter particular properties in comparison with the wild-type proteins. Chimeras of different CNG-channel subunits have already been described (Seifert R., Eismann E., Ludwig J., Baumann A., and Kaupp, U.B. (1999) Molecular determinants of a Ca^{2+}-binding site in the pore of cyclic nucleotide-gated channels: S5/S6 segments control affinity of intrapore glutamates. EMBO J., 18, 119-130).

Six genes for subunits of CNG channels are known in vertebrates (α1- α4, β1, β2). Additionally, there exist different isoforms of these subunits (Sautter A., Zonh X., Hofmann F., and Biel M. (1998) An isoform of the rod photoreceptor cyclic nucleotide-gated channel beta subunit expressed in olfactory. neurons. Proc. Natl. Acad. Sci. USA, 95, 4696-4701; Bönigk W., Bradley J., Müller F., Sesti F., Boekhoff I., Ronnett G.V., Kaupp U.B., and Frings S. (1999) The native rat olfactory cyclic nucleotide-gated channel is composed of three distinct subunits. J. Neurosci., 19, 5332-5347). The α1 subunit (Kaupp U.B., Niidome T., Tanabe T., Terada S., Bönigk W., Stühmer W., Cook N.J., Kangawa K., Matsuo H., Hirose T., Miyata T., and Numa S. (1989) Primary structure and functional expression from complementary DNA of the rod photoreceptor cyclic GMP-gated channel. Nature, 342, 762-766) and the β1 subunit (Chen T.Y., Peng Y.W., Dhallam R.S., Ahamed B., Reed R.R. and Yau K.W. (1993) A new subunit of the cyclic nucleotide-gated cation channel in retinal rods. Nature, 362, 764-767; Körschen H.G., Illing M., Seifert R., Sesti F., Williams A., Gotzes S., Colville C., Müller F., Dose

A., Godd M., Molday L., Kaupp U.Be., and Molday R.S. (1995) A 240 kDa protein represents the complete beta subunit of the cyclic nucleotide-gated cation channel from rod photoreceptor. Neuron, 15, 627-636) were first discovered in retinal rods, the α2 subunit (Bönigk W., Altenhofen W., Müller F., Dose A., Illing M., Molday R.S., and Kaupp U.B. (1993) Rod and cone photoreceptor cells express distinct genes for cGMP-gated channels. Neuron, 10, 865-877) and the β2 subunit (Gerstner A., Zong X., Hofmann F., and Biel M. (2000) Molecular cloning and functional characterization of a new modulatory cyclic nucleotide-gated channel subunit from mouse retina. J. Neurosci., 20, 1324-1332) in retinal cones, and the α3 subunit (Dhallan R.S., Yau K.W., Schrader K.A., and Reed R.R. (1990) Primary structure and functional expression of a cyclic nucleotide-activated channel from olfactory neurons. Nature, 347, 184-187; Ludwig J., Margalit T., Eismann E., Lancet D., and Kaupp U.B. (1990) Primary structure of cAMP-gated channel from bovine olfactory epithelium. FEBS Lett., 270, 24-29) and the α4 subunit (Bradley J., Li J., Davidson N., Lester H.A., and Zinn K. (1994) Heteromeric olfactory cyclic nucleotide-gated channels: a subunit that confers increased sensitivity to cAMP. Proc. Natl. Acad. Sci. USA, 91, 8890-8894; Liman E.R. and Buck L.B. (1994) A second subunit of the olfactory cyclic nucleotide-gated channel confers high sensitivity to cAMP. Neuron, 13,611-621) in olfactory cells of the nose.

In addition, CNG channels composed of said subunits were found in numerous other neuronal and non-neuronal cells and tissues (Richards M.J. and Gordon S.E. (2000) Cooperativity and cooperation in cyclic nucleotide-gated ion channels. Biochemistry, 39, 14003-14011). Moreover, CNG channels were found not only in vertebrates but also in nonvertebrates such as, for example, in Drosophila melanogaster (Baumann A., Frings

S., Godde M., Seifert R., and Kaupp U.B. (1994) Primary structure and functional expression of a Drosophila cyclic nucleotide-gated channel present in eyes and antennae. EMBO J., 13, 5040-5050) and plants (Leng Q., Mercier R.W., Yao W., and Berkowitz G.A. (1999) Cloning and first functional characterization of a plant cyclic nucleotide-gated cation channel. Plant Physiol., 121, 753-761). In principle, these and all other subunits of CNG channels can be modified according to the invention.

The genetically modified CNG channels may be used in cellular test systems as cAMP sensor for pharmacological studies,

(i) in order to study the action of ligands, agonists and antagonists on membrane-bound G protein-coupled receptors (GPCRs) which regulate intracellular cAMP concentration,

(ii) in order to study the action of activators and inhibitors on effector proteins (enzymes) which synthesize or hydrolyze cAMP,

(iii) in order to study the action of activators and inhibitors on other proteins which likewise intervene in the cAMP signal transduction cascade in a regulating manner, but also

(iv) in order to study the properties of GPCRs, effector proteins or other proteins involved in cAMP signal transduction cascades.

The proteins referred to as "membrane-bound G protein-coupled receptors" (GPCRs) according to the invention belong to the phylogenetically most varied, extremely extensive family of membrane-bound receptors (overview article: Morris A.J. and Malbon C.C. (1999) Physiological regulation of G protein-linked signaling. Physio. Rev., 79, 1373-1430): The family of GPCRs probably comprises distinctly more than 1 000 different members which can be classified on the basis of their

sequence similarity (Probst W.C., Snyder L.A., Schuster D.I., Brosius J., and Sealfon S.C. (1992) Sequence alignment of the G-protein coupled receptor superfamily. DNA Cell Biol., 11, 1-20) or based on the chemical nature of their natural ligands. A compilation and classification of the GPCRs known up to now can be found, for example, in the "GPCRDB" database (Horn F., Weare J., Beukers M.W., Horsch S., Bairoch A., Chen W., Edvardsen O., Campagne F., and Vriend G. (1998) GPCRDB: an information system for G protein-coupled receptors. Nucleic Acids Res., 26, 275-279). Some of the representatives of the individual classes are listed below in the form of an overview. Class A ("rhodopsin-like") includes rhodopsin itself and various sequence-related receptors which are classified on the basis of their natural ligands: these include receptors for (1) biogenic amines such as, for example, the muscarinic acetylcholine receptors, adrenergic receptors, dopamine receptors, histamine receptors, serotonin receptors, octapamine receptors, for (2) peptides, such as, for example, the angiotensin receptors, chemokine receptors, endotheline receptors, neuropeptide receptors, for (3) hormone proteins, such as, for example, FSH receptors, for (4) odorants, for (5) prostanoids, such as, for example, prostaglandin receptors, or for (6) nucleotides, such as, for example, adenosine receptors. Class B ("secretin-like") includes the secretin receptors themselves and, for example, receptors for calcitonin, glucagon, diuretic hormones or CRF (corticotropin-releasing factor). Class C ("metabotropic glutamate / pheromones") includes the metabotropic receptors themselves and also GABA-B receptors and others. Further classes comprise receptors from plants, fungi, insects, bacteria. All classes contain receptors whose function is not yet known or whose natural ligand is not yet known (orphan receptors). The natural ligands of each of about 200 different GPCR types are currently known and about a

further 100 GPCR types are orphan GPCRs. 700 or more GPCRs are presumably activated by odorants or tastants. It is possible in principle for all GPCRs regulating intracellular cAMP concentration to be coexpressed with the inventive genetically modified CNG channels as cAMP sensor in heterologous expression systems and for the action of ligands, agonists and antagonists to be studied pharmacologically.

It is also possible to study GCRPs which normally do not regulate intracellular cAMP concentration via stimulatory or inhibitory G proteins. GPCRs of this kind may be altered by genetic modification in such a way that they couple to the cAMP signal pathway. Said genetic modification may be carried out, for example, by preparing chimeric GCRPs (Liu J., Conklin B.R., Blin N., Yun J. and Wess J. (1995) Identification of a receptor-G-protein contact site critical for signaling specificity and G protein aviation. Proc. Natl. Acad. Sci. USA, 92, 11642-11646).

"Agonists" and "ligands" refer to substances which activate GCRP.

In contrast, "antagonists" refer to substances which, although being able to bind to GCRPs, cannot activate them. Antagonists inhibit the action of ligands or agonists in a dose-dependent manner.

"Effector proteins" which regulate intracellular cAMP concentration directly include adenylate cyclases and phosphodiesterases.

"Adenylate cyclases" whose activity is controlled in a GPCR-mediated manner are large, membrane-bound enzymes which catalyze the formation of cAMP from Mg^{2+} adenosine triphosphate and which are present in most cells, tissues and organs of the human body (Tang W.J. and Hurley J.H. (1998) Catalytic mechanism and regulation

of mammalian adenylyl cyclases. Mol. Pharmacol., 54, 231-240). Nine different classes of said adenylate cyclases are known altogether. Adenylate cyclases of this kind are also endogenously expressed in the cellular test systems of the invention and can be activated by heterologously expressed GPCRs and therefore play a decisive part in the functioning of the test system. Another class comprises soluble adenylate cyclases whose activity is presumably not regulated in a GPCR-mediated manner (Buck J., Sinclair M.L., Schapal L., Cann M.J., and Levin L.R. (2000) Cytosolic adenylyl cyclase defines a unique signaling molecule in mammals. Proc. Natl. Acad. Sci. USA, 96, 79-84). It is possible in principle to coexpress all adenylate cyclases with the inventive genetically modified CNG channels as cAMP sensor in heterologous expression systems and to study pharmacologically the action of inhibitors and activators.

"Phosphodiesterases" (PDEs) are enzymes inside the cell which hydrolyze cAMP and cGMP to give adenosine monophosphate (AMP) and guanosine monophosphate (GMP), respectively (Francis S.H., Turko I.V., and Corbin J.D. (2000) Cyclic nucleotide phosphodiesterases: relating structure and function. Prog. Nucleic Acid Res. Mol. Biol., 65, 1-52). Eleven different types of PDEs are known altogether. Some of said PDEs specifically hydrolyze cGMP, others in turn hydrolyze cAMP, and others again hydrolyze both cAMP and cGMP. Like GPCRs and adenylate cyclases, the PDEs are expressed in most cells, tissues and organs of the human body. In contrast to adenylate cyclases, however, only PDE6 which is specific for photoreceptors is activated in a GPCR-mediated manner. The activity of the other PDEs is instead regulated by various other mechanisms.

It is possible in principle to coexpress all PDEs which hydrolyze cAMP with the inventive genetically modified CNG channels as cAMP sensor in heterologous expression

systems and to study pharmacologically the action of inhibitors and activators.

"Activators" refers to substances which interact directly with adenylate cyclases or PDEs and thereby increase the enzymatic activity thereof.

"Inhibitors", in contrast, refer to substances which likewise interact directly with adenylate cyclases or PDEs but which reduce the activity thereof.

It is possible in principle for all proteins which regulate intracellular cAMP concentration to be coexpressed with the inventive genetically modified CNG channels as cAMP sensor in heterologous expression systems and to be studied pharmacologically.

The proteins to be studied may be expressed in heterologous systems either transiently or, preferably, stably.

"Transient expression" means that the heterologously expressed protein is expressed by the cells of the expression system only for a defined period of time.

"Stable expression" means that the introduced gene is stably integrated into the genome of the cells of the heterologous expression system. The new cell line produced in this way expresses the corresponding protein in each subsequent cell generation.

For expression, the cDNA coding for the protein to be studied is cloned into an expression vector and transformed into the cells of a suitable expression system.

"Expression vectors" refer to any vectors which can be used for introducing ("transforming") cDNAs into the appropriate cell lines and functionally expressing there the corresponding proteins ("heterologous expression"). Preferably, the transformation may be carried out using the pcDNA vectors (Invitrogen).

Suitable "heterologous expression systems" are in principle all eukaryotic cells such as, for example, yeast, Aspergillus, insect, vertebrate and in particular mammalian cells. Examples of suitable cell lines are CHO (Chinese hamster ovary) cells, for example the K1 line (ATCC CCL 61), including the Pro 5 variant (ATCC CRL 1781), COS cells (African green monkey), for example the CV-1 ceu line (ATCC CCL 70), including the COS-1 variant (ATCC CRL 1650) and the COS-7 variant (ATCC 1651), BHK (baby hamster kidney) cells, for example the line BHK-21 (ATCC CCL 10), MRC-5 (ATCC CCL 171), murine L cells, murine NIH/3T3 cells (ATCC CRL 1658), murine C127 cells (ATCC CRL-1616), human carcinoma cells such as, for example, the HeLa line (ATCC CCL 2), neuroblastoma cells of the lines IMR-32 (ATCC CCL 127), neuro-2A cells (ATCC CLL 131), SK-N-MC cells (ATCC HTB 10) and SK-N-SH cells (ATCC HTB 11), PC12 cells (ATCC CRL 1721), and Sf9 cells (Spodoptera frugiperda) (ATCC CRL 1711). Preference is given to using HEK 293 (human embryonic kidney) cells (ATCC CRL 1573), including the SF variant (ATCC 1573.1). Particular preference is given to the cell line prepared according to the invention, DSM ACC 2516.

The action of test substances on the protein to be studied can be measured using a fluorescence-optical measurement method.
The protein to be studied is heterologously expressed together with cAMP sensor of the invention in a cellular test system and activated or inhibited by ligands, agonists, antagonists, activators or inhibitors. The cAMP sensor registers the changes in cAMP concentration and Ca^{2+} ions flow into the cell to a larger or reduced extent.

"Fluorescence-optical measurement methods" means that the change in intracellular Ca^{2+} concentration is made visible using a fluorescent Ca^{2+} indicator. By now, a

multiplicity of different Ca^{2+} indicators are known (Haugland R.P., (1996) Handbook of fluorescent probes and research chemicals. Molecular Probes Inc.). They include, for example, Fluo-3, Calcium Green, Fura-2 and Fluo-4 (Molecular Probes), the latter being preferred according to the invention. Since indicators of this kind are usually water-soluble and therefore cannot pass the hydrophobic lipid membrane of cells, the indicators are instead applied in the form of an acetoxy methyl ester compound (Tsien R.Y. (1981) A non-disruptive technique for loading calcium buffers and indicators into cell. Nature, 290, 527-528). These compounds, in contrast, are hydrophobic and are taken up by the cells. Inside the cell, the ester bond is cleaved by endogenous, intracellular esterases and the indicator is again present in its water-soluble form in which it remains in the cell interior where it accumulates and can thus be used as intracellular Ca^{2+} indicator. This indicator, when excited with light of a suitable wavelength, then shows fluorescence which depends on the intracellular Ca^{2+} concentration. The degree (amplitude) of fluorescence and the time course (kinetics) correlate with the degree and the time course of activation of the protein studied and can be monitored in real time using fluorescence detectors with a very good signal-to-noise ratio and plotted using a suitable software.

Likewise, the aequorin protein complex which consists of apoaequorin and the chromophoric cofactor coelenterazine or comparable complexes may be used as Ca^{2+} indicators for measuring intracellular Ca^{2+} concentration (Brini M., Pinton P., Pozzan T. and Rizzuto R. (1999) Targeted recombinant aequorins: tools for monitoring (Ca^{2+}) in the various compartments of a living cell. Micrsc. Res. Tech., 46, 380-389). For this purpose, apoaequorin must be heterologously expressed together with the cAMP sensor of the invention and the

protein to be studied in the cellular test system. Prior to the measurements, the cells must be incubated with coelenterazine so that apoaequorin and coelenterazine can assemble to give the active aequorin complex. When the intracellular Ca^{2+} concentration increases, coelenterazine is oxidized to coelenteramide. In this process, CO_2 is formed and luminescence is emitted. Disadvantageously, said process is irreversible. Said luminescence may be registered using a suitable optical detector (luminescence-optical measurement method). Although this optical measurement method has a similar sensitivity to the fluorescence-optical measurement method, it is however less suitable for measurements in which the course of the reaction is followed in real time.

Fluorescence- or luminescence-optical measurements using a test system of the invention may be carried out in cuvette measuring devices, in Ca^{2+} imaging microscopes or in fluorescence or luminescence readers.

According to the invention, preference is given to carrying out the measurements in wells of plastic containers (multiwell plates) in fluorescence readers. The cells may be introduced in suspension or else, preferably, attached to the bottom of said wells. Multiwell plates having a different number of wells may be used, such as, for example, multiwell plates having 96, 384, 1536 or more wells. Said multiwell plates make it possible to carry out a multiplicity of identical or different measurements in a single plate.

"Fluorescence reader" or "luminescence reader" are very sensitive optical measuring devices which can be used to measure fluorescence or luminescence in multiwell plates. It is possible to study in such devices the

action of ligands, agonists, antagonists, activators or inhibitors very rapidly and with a high throughput.

According to the invention, it is possible to study the properties of GPCRs, effector proteins or other proteins involved in cAMP signal transduction cascades quantitatively using fluorescence- or luminescence-optical measurements.

However, it is also possible in principle, according to the invention, to carry out measurements with a high throughput of tests per day. The search for new pharmacological active compounds is thus possible. It is possible here to test up to 100 000 substances per day (high throughput screening, HTS screening) or more than 100 000 substances per day (Ultra-HTS screening, UHTS screening).

Using a FLIPR384 fluorescence reader (Molecular Devices), for example, it is possible to carry out up to 384 independent measurements simultaneously and to monitor fluorescence in real time.

The invention is described in more detail below on the basis of the exemplary embodiments and the attached figures:

Examples 1-4

Genetically modified, homooligomeric bovine α3 CNG channels were prepared, whose sequence has, in place of threonine T537 of the wild-type channel, a methionine (T537M) or valine (T537V).

For this purpose, the nucleic acid coding for the α3 subunit of the bovine CNG channel (SEQ ID NO 1) was altered in position 537 by site-specific mutagenesis using molecular-biological methods ("genetically

modified") so that a nucleic acid coding for the T537M mutant (SEQ ID NO 3) and another one coding for the T537V mutant (SEQ ID NO 5) were generated (see Methods).

The SEQ ID NO 2, 4 and 6 depict the amino acid sequences of the wild-type channel, the T537M mutant and the T537V mutant, respectively.

The sensitivity and selectivity of the T537M mutant and of the T537V mutant for cAMP and cGMP were determined by electrophysiological methods (see Methods) and compared with the properties of the wild-type channel. For this purpose, the corresponding nucleic acids were cloned into the expression vector pcDNA3.1 (Invitrogen) (see Methods). The expression constructs were then used to transform the cells of the human embryonic kidney cell line 293 (HEK293 cells) and the corresponding proteins were functionally expressed therein either transiently or stably (see Methods). The results of the studies are depicted in figures 1-4.

The figures 1A, 2A, 3A and 4A depict in each case the current-voltage (IV) relationship of the heterologously expressed genetically modified CNG channels in the presence of various concentrations of cAMP (1A, 3A) and cGMP (2A, 4A), respectively. They depict in each case a current-voltage relationship typical for CNG channels in the presence of cAMP and cGMP, respectively, and confirm that the channels are functionally expressed in the HEK293 cells.

Figures 1-4 B depict the dependence of the average current on the cAMP concentration and cGMP concentration, respectively, in each case in the form of a dose-response relationship. The results of these measurements were used to calculate the sensitivity of the CNG channels to cAMP and cGMP. The concentration of cAMP and cGMP with V_m = +100 mV, at which said channels conduct half of the possible maximum current ($K_{1/2}$ value) is used as measure for the sensitivity of said

CNG channels. The lower $K_{1/2}$, the higher the sensitivity of the channel to the corresponding cyclic nucleotide. Figures 1B (T537M mutant) and 3B (T537V mutant) depict in each case the dose-response relationship for cAMP; figure 2B (T537M mutant) and 4B (T537V mutant), on the other hand, indicate in each case the dose-response relationship for cGMP.

In figures 1C (T537M mutant) and 3C (T537V mutant), in each case the normalized dose-response relationship of the mutants is compared with that of the α3 wild-type channel for cAMP; in figures 2C (T537M mutant) and 4C (T537V mutant), on the other hand, in each case the normalized dose-response relationship is compared with that of the α3 wild-type channel for cGMP.

The table below summarizes the sensitivity properties of the genetically modified CNG channels and the wild-type channel. The $K_{1/2}$ values for cAMP and for cGMP are listed.

	$K_{1/2}$ for cAMP	$K_{1/2}$ for cGMP
1. Wild type	80 μM	1.6 μM
2. T537S	14 μM	0.7 μM
3. **T537M**	**2.7 μM**	**14.9 μM**
4. **T537V**	**34 μM**	**241 μM**

Half-maximum activation of the T537M mutant, the T357V mutant and the wild type is produced by 2.7 μM cAMP (fig. 1C), 34 μM cAMP (fig. 3C) and 80 μM cAMP (fig. 1C, 3C), respectively. The T537M mutant and the T547V mutant are about 30 times and 3 times, respectively, more sensitive to cAMP in comparison with the wild type. In comparison with the T537S mutant, the T537M mutant is about 5 times more sensitive to cAMP, while the T537V mutant is about 2.5 times less sensitive to cAMP than the T537S mutant.

Half-maximum activation of the T537M mutant, the T537V mutant and the wild type is produced by 14.9 μM cGMP

(fig. 2C), 241 μM cGMP (fig. 4C) and 1.6 μM cGMP (fig. 2C, 4C), respectively. Thus, the T537M mutant and the T537V mutant are about 9 times and about 150 times, respectively, less sensitive to cGMP in comparison with the wild type. In comparison with the T537S mutant, the T537M mutant is 21 times less sensitive to cGMP and the T537V mutant is even about 350 times less sensitive to cGMP.

The selectivity of a CNG channel for cAMP or cGMP is obtained by comparing the $K_{1/2}$ values. Example: The T537M mutant has a $K_{1/2}$ of 2.7 μM for cAMP and a $K_{1/2}$ of 14.9 μM for cGMP. This means that half-maximum activation of said mutant is produced by as low as 2.7 μM cAMP but only by 14.9 μM cGMP. According to this, the mutant is markedly more sensitive to cAMP than to cGMP. The quotient (14.9 μM/2.7 μM) indicates the relative selectivity. Thus, the T537M mutant is about 6 times more selective for cAMP. The T537V mutant is likewise about 6 times more selective for cAMP. The T537S mutant, on the other hand, is about 20 times and the wild-type channel even about 50 times more selective for cGMP.

The results of the measurements show the following: CNG-channel mutants whose (1) absolute cAMP sensitivity is very much higher than that of the wild-type channels were generated and identified, and (2) the selectivity for cAMP and cGMP is reversed. The genetic modifications impart an enormous selective sensitivity to cAMP to these CNG channels. In addition, the two genetically modified CNG channels are so insensitive to cGMP that even relatively large changes in intracellular cGMP concentration do not interfere with the cAMP concentration measurement. Specifically, the T537M mutant which is preferred according to the invention is particularly suitable as cellular cAMP sensor. It may be used, for example, in cellular test

systems for studying the action of substances which can influence the intracellular cAMP concentration and renders such test systems usable in practice in the first place: a sensor of this kind may then be used to register reliably and with a very good signal-to-noise ratio and monitor in real time even those slight changes in intracellular cAMP concentration as are triggered, for example, by the activation of GPCRs.

Methods

Preparation of genetically motivated CNG channels by site-specific mutagenesis

The cDNA for the α3 subunit of the bovine CNG channel was excised with the aid of suitable restriction endonucleases (Eco RV and Nsi I) from the plasmid pCHOLF102 (Altenhof W., Ludwig J., Eismann E., Kraus W., Bönigk W. and Kaupp U.B. (1991) Control of ligand specificity in cyclic nucleotide-gated channels from rod photoreceptors and olfactory epithelium. Proc. Natl. Acad. Sci. USA, 88, 9868-9872) and cloned into pcDNAlamp (Invitrogen). The plasmid was referred to as pcA-bolf. The cDNA fragment was then cloned via Eco RV and Xba I into pcDNA3 derivatives. The plasmid was referred to as pc3-bolf. The genetically modified α3 subunit of the CNG channel was prepared by means of site-specific mutagenesis (Herlitze S. and Koenen M. (1990). A general and rapid mutagenesis method using polymerase chain reaction. Gene, 91, 143-147).

The mutagenesis primer for preparing the T537M-α3 subunit had the following sequence (SEQ ID NO 7):

5'-CGACGCATGGCGAACATCCGCAGTCT-3'

The mutagenesis primer for preparing the T537V-α3 subunit had the following sequence (SEQ ID NO 8):

5'-CGACGCGTCGCGAACATCCGCAGTCT-3'

First, a PCR of pc3-bolf was carried out using the mutagenesis primer and the counterprimer #1817 (5'-TTGGCTGCAGCTATTATGGCTTCTCGGCAG-3') (SEQ ID NO 9). A 100-µl PCR mixture comprised 10 ng of template DNA, 1 × PCR buffer of Taq polymerase incl. 1.5 mM of $MgCl_2$, 200 µM of dNTPs, 1 U of Taq polymerase and in each case 150 ng of the two primers. The PCR conditions were as follows. 2 minutes of denaturation at 94°C, followed by 25 cycles of in each case 45 s at 94°C, 45 s at 46°C, 45 s at 72°C. The 404 bp fragment was purified and used together with the overlapping 666 bp Sma I/Pvu II restriction fragment as template for a further PCR. The flanking primers #1813 (5'-GTCGGATCCTCCACACTCAAGAAAGTG-3') (SEQ ID NO 10) and #1817 were used for amplification. The PCR mixture had the same composition as indicated above, with the template used being 250 ng of the 1st PCR fragment and 10 ng of the restriction fragment instead of 10 ng of plasmid DNA. The PCR fragment was cleaved with BsrGI and substituted for the corresponding fragment in pc3-bolf. The sequence of the altered DNA section was checked by sequencing.

Isolation of cDNA clones

The CRF receptor was cloned by carrying out a PCR of primary strand cDNA of rat hypophyses, using the primers #843 (5'-AGCGGGATCCACCATGGGACGGCGCCCGCA-3') (SEQ ID NO 11) and #842 (5'-GGCCTGGAGCTCACACTG-3') (SEQ ID NO 12). A 100 µl PCR mixture comprised 10 ng of primary strand cDNA, 1 × PCR buffer for Taq polymerase incl. 1.5 mM of $MgCl_2$, 200 µM of dNTPs, 1 U of Taq polymerase and in each case 150 ng of the two primers. The PCR conditions were as follows. 2 minutes of denaturation at 94°C, followed by 44 cycles of in each case 45 s at 94°C, 45 s at 56°C, 75 s at 72°C. The 1271 bp fragment was cloned via BamHI and SacI in pBluescript SK^- into (pBCRFR1). The sequence

corresponds to the published sequence L25438 (Chang, C.P., Pearse R.V. II, O'Connel S. and Rosenfed M.G. (1993). Identification of a seven transmembrane helix receptor for corticotropin-releasing factor and sauvagine in mammalian brain. Neuron, 11, 1187-1195). The cDNA was subcloned into a pcDNA derivative for heterologous expression (pcCRFR1).

The plasmid pcK_1M which contains the cDNA of the dopamine receptor from Drosophila (Gotzes F., Balfanz S. and Baumann A. (1994) Primary structure and functional characterization of a Drosophila dopamine receptor with high homology to human D1/5 receptors. Receptors Channels, 2, 131-141), was kindly provided by Dr. F. Gotzes.

Heterologous expression in HEK 293 cells and preparation of stable cell lines

Transient expression in HEK 293 cells was carried out as described in Baumann A., Frings S., Godde M., Seifert R. and Kaupp U.B. (1994) Primary structure and functional expression of a Drosophila cyclic nucleotide-gated channel present in eyes and antennae. EMBO J., 13, 5040-5050. For electrophysiological characterization, the cells were transferred to glass slides which had been coated with poly L-lysine, on the day after transfection. The electrophysiological studies were then carried out on the following day.

To prepare the stable cell lines, the cells were transfected in the same manner. Cell clones stably expressing the desired gene were selected by seeding 2×10^4 cells on a 9 cm cell culture dish on the day after transfection. The cells were cultured for 20 days, with either G418 (800 µg/ml) (Calbiochem), Zeocin (100 µg/ml) (Invitrogen) or Hygromycin (100 µg/ml) (Invitrogen) being added to the cell culture medium. After 20 days, the cell clones expressing the

resistance gene were isolated and expression of the CNG-channel gene or of the receptor gene was checked by Western blot analyses and functional studies (electrophysiological measurement and fluorescence-optical measurements). For Western blot analysis, the cells were homogenized in a lysis buffer (10 mM Hepes, 1 mM DTT and 1 mM ETDA at pH 7.4), 5×shock-frozen (in liquid nitrogen) and finally centrifuged at 55 000 rpm for 10 min. The membrane pellet was resuspended in dissolving buffer (150 mM NaCl, 1 mM $MgCl_2$, 20 mM Hepes at pH 7.5, 0.1 mM EGTA and 0.5% Triton X-100). In each case 3 µg of membrane proteins were separated by means of SDS-PAGE, transferred to Immobilon membranes and labeled with specific antibodies. The immunoreactivity was made visible with the aid of the ECL detection kit (Amersham).

Double-stable cell lines were prepared by continuing culturing a cell clone which stably expressed either the CNG-channel gene or the receptor gene and using it for transfecting the in each case other cDNA. The cell clones were selected and chosen as described above.

The fluorescence-optical measurements partly involved using cells which stably expressed a genetically modified α3 subunit of the CNG channel and transiently expressed the Drosophila dopamine receptor. For this purpose, cells of the stable T537M cell line were transfected as described by Baumann A., Frings S., Godde M., Seifert R., and Kaupp U.B. (1994) Primary structure and functional expression of a Drosophila cyclic nucleotide-gated channel present in eyes and antennae. EMBO J., 13, 5040-5050. On the day after transfection, the cells were seeded into a multiwell plate with 96 wells. The cell density per well was 2×10^4 cells.

For the fluorescence-optical measurement of stable cell lines, the cells were seeded into said multiwell plate

with 96 wells one or two days before the measurement. The cell density was from 1.5 to 4×10^4 cells.

Electrophysiology

The genetically modified and the wild-type GNG channels were in each case heterologously expressed in HEK 293 cells (Baumann A., Frings S., Godde M., Seifert R., and Kaupp U.B. (1994) Primary structure and functional expression of a Drosophila cyclic nucleotide-gated channel present in eyes and antennae. EMBO J., 13, 5040-5050). The electrophysiological characterization of the genetically modified CNG channels was carried out using the patch clamp technique under voltage clamping conditions. The activation properties of the channels were determined and compared to those of the wild-type channels (figures 1 to 4):
inside-out patches were excised from cells which stably expressed genetically modified or wild-type CNG channels. The bath solution containing various concentrations of cyclic nucleotides was used to flow over the membrane patches. Starting from a holding voltage of 0 mV, sudden voltage changes were applied to various test voltages between -100 mV and +100 mV at the patch with a step width of 20 mV. The currents were registered and analyzed using standard methods.

Figure 1A depicts the current-voltage (IV) relationship of the depicts T537M-α3 subunit of the bovine CNG channel. The average currents (I) at various cAMP concentrations are plotted as a function of the voltage (Vm): 0 µM (filled circles), 0.3 µM (open circles), 1 µM (filled triangles), 3 µM (open triangles), 10 µM (filled squares), 30 µM (open squares), 100 µM (filled diamonds).

Figure 1B shows the dependence of the average currents on cAMP concentration for the T537M-α3 subunit of the

bovine CNG channel at a voltage of +100 mV (filled circles). The continuous line has been calculated according to the Hill equation $I = (I_{max}-I_{min})c^n/(K^n+c^n) + I_{min}$ (I: current; I_{max}: maximum current; I_{min}: minimum current; $K_{1/2}$: concentration at which half-maximum activation of the channels occurs; n: Hill coefficient; c: cAMP concentration) with the following parameters: I_{max} = 328 pA; I_{min} = 24 pA; $K_{1/2}$ = 2.9 µM; n = 2.4.

Figure C depicts the normalized dose-response relationship of the T537M-α3 subunit of the bovine CNG channel (continuous line) and that of the wild-type bovine α3 subunit (dotted line) at +100 mV. The continuous and dotted lines have been calculated according to the normalized Hill equation $I_{norm.} = c^n/(c^n + K^n)$ with the following averaged parameters: (continuous line: $K_{1/2}$ = 2.7 µM; n = 2.4; dotted line: $K_{1/2}$ = 80 µM; n = 2.0).

Figure 2A depicts the current-voltage (IV) relationship of the heterologously expressed T537M-α3 subunit of the bovine CNG channel. The average currents (I) at various cGMP concentrations are plotted as a function of the voltage (Vm): 0 µM (filled circles), 1 µM (open circles), 3 µM (filled triangles), 10 µM (open triangles), 30 µM (filled squares), 100 µM (open squares), 300 µM (filled diamonds), 2 000 µM (open diamonds).

Figure 2B shows the dependence of the average currents on cGMP concentration for the T537M-α3 subunit of the bovine CNG channel at a voltage of +100 mV (filled circles). The continuous line has been calculated according to the Hill equation $I = (I_{max}-I_{min})c^n/(K^n+c^n) + I_{min}$ (I: current; I_{max}: maximum current; I_{min}: minimum current; $K_{1/2}$: concentration at which half-maximum activation of the channels occurs; n: Hill coefficient;

c: cGMP concentration) with the following parameters: I_{max} = 167 pA; I_{min} = 11 pA; $K_{1/2}$ = 12.0 µM; n = 2.0.

Figure 2C depicts the normalized dose-response relationship of the T537M-α3 subunit of the bovine CNG channel (continuous line) and that of the wild-type bovine α3 subunit (dotted line) at +100 mV. The continuous and dotted lines have been calculated according to the normalized Hill equation $I_{norm.} = c^n/(c^n + K^n)$ with the following averaged parameters: (continuous line: $K_{1/2}$ = 14.9 µM; n = 1.9; dotted line: $K_{1/2}$ = 1.6 µM; n = 2.0).

Figure 3A depicts the current-voltage (IV) relationship of the heterologously expressed T537V-α3 subunit of the bovine CNG channel. The average currents (I) at various cAMP concentrations are plotted as a function of the voltage (Vm): 0 µM (filled circles), 3 µM (open circles), 10 µM (filled triangles), 30 µM (open triangles), 100 µM (filled squares), 300 µM (open squares), 1 000 µM (filled diamonds).

Figure 3B shows the dependence of the average currents on cAMP concentration for the T537V-α3 subunit of the bovine CNG channel at a voltage of +100 mV (filled circles). The continuous line has been calculated according to the Hill equation $I = (I_{max}-I_{min})c^n/(K^n+c^n) + I_{min}$ (I: current; I_{max}: maximum current; I_{min}: minimum current; $K_{1/2}$: concentration at which half-maximum activation of the channels occurs; n: Hill coefficient; c: cAMP concentration) with the following parameters: I_{max} = 181 pA; I_{min} = 11 pA; $K_{1/2}$ = 23.4 µM; n = 2.2.

Figure 3C depicts the normalized dose-response relationship of the T537V-α3 subunit of the bovine CNG channel (continuous line) and that of the wild-type bovine α3 subunit (dotted line) at +100 mV. The continuous and dotted lines have been calculated

according to the normalized Hill equation $I_{norm.} = c^n/(c^n + K^n)$ with the following averaged parameters: (continuous line: K = 34 µM; n = 1.5; dotted line: $K_{1/2}$ = 80 µM; n = 2.0).

Figure 4A depicts the current-voltage (IV) relationship of the heterologously expressed T537V-α3 subunit of the bovine CNG channel. The average currents (I) at various cGMP concentrations are plotted as a function of the voltage (Vm): 0 µM (filled circles), 30 µM (open circles), 100 µM (filled triangles), 300 µM (open triangles), 1 000 µM (filled squares), 2 000 µM (open squares).

Figure 4B shows the dependence of the average currents on cGMP concentration for the T537V-α3 subunit of the bovine CNG channel at a voltage of +100 mV (filled circles). The continuous line has been calculated according to the Hill equation $I = (I_{max}-I_{min})c^n/(K^n+c^n) + I_{min}$ (I: current; I_{max}: maximum current; I_{min}: minimum current; $K_{1/2}$: concentration at which half-maximum activation of the channels occurs; n: Hill coefficient; c: cGMP concentration) with the following parameters: I_{max} = 1 389 pA; I_{min} = 13 pA; $K_{1/2}$ = 255.2 µM; n = 2.2.

Figure 4C depicts the normalized dose-response relationship of the bovine T537V-α3 subunit (continuous line) and that of the wild-type bovine α3 subunit (dotted line) at +100 mV. The continuous and dotted lines have been calculated according to the normalized Hill equation $I_{norm.} = c^n/(c^n + K^n)$ with the following averaged parameters: (continuous line: K = 241 µM; n = 2.1; dotted line: $K_{1/2}$ = 1.6 µM; n = 2.0).

Fluorescence-optical measurements

The fluorimetric measurements of intracellular Ca^{2+} concentrations were carried out in multiwell plates with 96 wells (fig. 5A-5G). The cells were loaded with the Ca^{2+}-sensitive fluorescent dye Fluo-4 one hour before the measurement. The loading solution contained 120 mM of NaCl, 3 mM of KCl, 50 mM of glucose, 10 mM of Hepes (pH 7.4), 3 mM of $MgCl_2$ and 4 µM of Fluo4-AM (molecular probes). The measuring solution contained 120 mM of NaCl, 3 mM of KCl, 50 mM of glucose, 10 mM of Hepes (pH 7.4) and 3 mM of $CaCl_2$. The time course of the change in fluorescence intensity after application of agonists, antagonists, enzyme activators or enzyme inhibitors was registered using a fluorescence reader (FLUOstar, BMG-Labtechnologies). The excitation wavelength was 485 nm. The emission wavelength was 520 nm.

Example 5

Figure 5A depicts the fluorimetrically measured change in intracellular Ca^{2+} concentration in cells transiently expressing the Drosophila dopamine receptor (DR) (Gotzes F., Balfanz S., and Baumann A. (1994) Primary structure and functional characterization of a Drosophila dopamine receptor with high homology to human D1/5 receptors. Receptor Channels, 2, 131-141) and stably expressing the T537M mutant of the α3-CNG channel. The time course of fluorescence intensity (RFU: relative fluorescence unit) after stimulation of the cells with different concentrations of the agonist dopamine is shown. The arrow marks the point in time at which the cells were stimulated with dopamine. The dopamine-induced activation of the dopamine receptor first leads in the cell to activation of a stimulatory G protein and finally to activation of an adenylate cyclase which synthesizes cAMP. Binding of cAMP opens CNG channels and Ca^{2+} ions flow into the cell. The speed and extent of the change in Ca^{2+} concentration depends

the dopamine concentration. The dopamine concentration was 25 nM (filled circles), 50 nM (open circles), 400 nM (filled triangles) or 600 nM (open triangles). For comparison, cells which do not express the dopamine receptor were also stimulated with 25 nM dopamine (filled rectangles).

Example 6

Figure 5B depicts the fluorimetrically measured change in intracellular Ca^{2+} concentration in cells which stably express both the corticotropin-releasing factor (CRF) receptor and the T537M mutant of the α3-CNG channel. The time course of fluorescence intensity after stimulation of the cells with the agonist CRF is shown. The arrow marks the time at which the cells were stimulated with CRF. CRF-induced activation of the CRF receptor leads in the cell first to activation of a stimulatory G protein and finally to activation of the adenylate cyclase which synthesizes cAMP (see, for example: Eckart K., Radulovic J., Radulovic M., Jahn O., Blank T., Stiedl O., and Spiess J. (1999) Actions of CRF and its analogs. Curr. Med. Chem., 6, 1035-1053; Perrin M.H. and Vale W.W. (1999). Corticotropin releasing factor receptors and their ligand family. Ann. N.Y. Acad. Sci., 885, 312-328). Binding of cAMP opens CNG channels and Ca^{2+} ions flow into the cell. The speed and extent of the change in Ca^{2+} depend on the CRF concentration. The CRF concentration was 100 pM (filled triangles), 300 pM (open circles) or 1 000 pM (filled circles).

Figures 5A and 5B depict by way of example for two different GCRPs coupling to a stimulatory G protein that the method of the invention can be used to measure the action of agonists on said GCRPs with high sensitivity. The method is equally suitable for all other GCRPs coupling to stimulatory G proteins. The

examples of figure 5A and 5B also show that it is possible to express for the method of the invention the heterologously expressed proteins (genetically modified α subunit of the CNG channel and GCRP) both transiently and stably in the cells.

Example 7

Figure 5C indicates that the method is suitable for quantitatively determining the effectiveness of different antagonists. Cells which stably express the corticotropin-releasing factor (CRF) receptor and the T537M mutants of the α3-CNG channel were treated with the helical antagonist 9-41 (Rivier J., Rivier C., and Vale W.W. (1984) Synthetic competitive antagonists of corticotropin-releasing factor: effect on ACTH secretion in the rat. Science, 224, 889-891), before being stimulated with CRF (1 nM). The time course of fluorescence intensity is shown. The arrow marks the point in time at which the cells were stimulated with CRF. With the same CRF concentration, the speed and extent of the change in Ca^{2+} depend on the concentration of the antagonist. The concentration of the antagonist was 0 nM (filled circles), 10 nM (open circles) or 100 nM (filled triangles).

Example 8

Figure 5D indicates that the method is suitable for measuring the effectiveness of particular enzyme activators. The effect of an activator of adenylate cyclase (AC) on the change in intracellular Ca^{2+} concentration is shown. Cells which stably express the T537M mutants of the α3-CNG channel were stimulated with different concentrations of the AC activator forskolin. The time course of fluorescence intensity is shown. The arrow marks the point in time at which the cells were stimulated with forskolin. Forskolin

directly activates the adenylate cyclase endogenous to said cells (Seamon K.B. and Daly J.W. (1981) Forskolin: a unique diterpene activator of cyclic AMP-generating systems. J. Cyclic Nucleotide Res., 7, 201-224).

The rate and extent of the change in intracellular Ca^{2+} concentration depend on the concentration of the AC activator. The forskolin concentration was 0.5 µM (open triangles), 0.75 µM (filled triangles), 2 µM (open circles) or 4 µM (filled circles).

Example 9

Figure 5E indicates that the method is suitable for determining the effectiveness of particular enzyme inhibitors. The effect of a phosphodiesterase (PDE) inhibitor on the forskolin-induced change in intracellular Ca^{2+} concentration is shown. Cells which stably express the T537M mutants of the α3-CNG channel were first treated with different concentrations of the PDE inhibitor IBMX and then stimulated with 5 µM forskolin. The plot shows the time course of fluorescence intensity. The arrow marks the point in time at which the cells were stimulated with forskolin. The PDE inhibitor inhibits the activity of the endogenous PDEs and thereby reduces degradation of cAMP synthesized due to a stimulation of the endogenous AC. The extent of the change in intracellular Ca^{2+} concentration depend on the concentration of the PDE inhibitor. The IBMX concentration was 0 µM (filled circles), 10 µM (open circles), 50 µM (filled triangles) or 100 µM (open triangles).

The example shows that the method is suitable for measuring sensitively the effectiveness of PDE inhibitors. It is possible to study inhibitors of endogenous PDEs but also inhibitors of heterologously expressed PDEs.

The method of the invention is also suitable for determining the activity of GCRPs coupling to the inhibitory G protein G_i. If GCRPs of this kind are activated, the cellular cAMP concentration is reduced because ACs are inhibited. For the method of the invention, the cells must first be stimulated to synthesize cAMP (e.g. by the AC activator forskolin), before the activity of agonists can then be measured. Simultaneous or sequential application of antagonists and agonists make it also possible to test the effectiveness of antagonists for such GCRP.

The method is analogously suitable for determining any substances capable of increasing or reducing the cAMP concentration in the cell in any way. The direct points of attack of these substances (e.g. GCRP or enzymes) may either be present endogenously in the cells or may be transiently or stably expressed in cells together with genetically modified CNG channels.

Example 10

Figure 5F(1) indicates that the method makes also quantitative analyses possible. Cells which stably express an endogenous adenosine receptor (Cooper J., Hill S.J. and Alexander S.P. (1997). An endogenous A2B adenosine receptor coupled to cyclic AMP generation in human embryonic kidney (HEK 293) cells, Br. J. Pharmacol., 122, 546-550) and the T537M mutant of the α3-CNG channel were stimulated with different concentrations of adenosine. The time course of fluorescence intensity is shown. The arrow marks the point in time at which the cells were stimulated with adenosine. The adenosine-induced activation of the adenosine receptor leads in the cell first to activation of a stimulatory G protein and finally to activation of the adenylate cyclase which synthesizes cAMP. Binding of cAMP opens CNG channels and Ca^{2+} ions flow into the cell. The extent and rate of the change

in intracellular Ca^{2+} concentration depend on the adenosine concentration. The adenosine concentration was 0.9 µM (filled circles) and 1.5 µM (open circles), 3 µM (filled triangles), 6 µM (open triangles), 25 µM (filled rectangles) or 37.5 µM (open rectangles).

Figure 5F(2) shows the dose-response relationship for adenosine. The rate of the change in fluorescence after addition of adenosine (initial rate) correlates with the adenosine concentration. The initial rates are plotted as a function of adenosine concentration. The continuous line has been calculated according to the Hill equation: initial slope = $a \times c^n/(K^n+c^n)_n$ (c = adenosine concentration; K: concentration at which the half-maximum initial rate is reached; n: Hill coefficient; a = maximum initial slope) with the following parameters: a = 1 403 RFU/s; K = 5.15 µM; n = 2.16),

The $K_{1/2}$ value determined in this way agrees very well with the value of 5 µM indicated in the literature (Peakman M.C. and Hill S.J. (1994) Adenosine A2B receptor-mediated cyclic AMP accumulation in primary rat atrocytes, Br. J. Pharmacol., 111, 191-198).

Example 11

Figure 5G demonstrates the enormous improvement in sensitivity of the measuring system due to the use of the modified subunit of the α3-CNG channel. Cells which stably express the T537M mutant of the α3-CNG channel were stimulated with 3 µM adenosine to about half maximum (open circles) or with 25 µM adenosine to maximum (filled circles). The extent of the change in intracellular Ca^{2+} concentration (fluorescence signal) was plotted as a function of time. The arrow marks the point in time at which the cells were stimulated with adenosine. Even after stimulation with half-maximum adenosine concentration a distinct increase in the fluorescence signal can be observed. In contrast, the

cells which stably express the wild-type α3-CNG channel show no increase in the fluorescence signal, even after maximum stimulation with 25 μM adenosine (filled triangles). Cells which stably express the T537S mutant of the α3-CNG channel were also stimulated for comparison. After maximum stimulation of the endogenous adenosine receptor, only a very low increase in the fluorescence signal can be observed (filled rectangles).

The use of the genetically modified subunits of the invention considerably increases the sensitivity of the intracellular test system for studying the action of substances influencing the cAMP signal pathway, only thereby making cell sensitivity usable in practice. The signals are so large and the signal-to-noise ratio is so good that even quantitative analyses are possible.

Suitable for preparing the cell lines of the invention are (not only HEK 293 cells) but in principle any eukaryotic cells which can be cultured in cell cultures. Both adherently growing cells and cells growing in suspensions may be used.

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<120> Genetically modified cyclic nucleotide-controlled ion channels and use thereof

<130> Application FZJ-0102

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FZJ 0102 PCT

What is claimed is:

1. A genetically modified cyclic nucleotide-gated ion channel (CNG channel) composed of subunits which have been modified at the position corresponding to threonine T537 in the bovine α3 subunit so that they have higher sensitivity for cAMP and/or higher selectivity for cAMP than for cGMP in comparison with the wild type according to SEQ ID NO 1 and 2.

2. The genetically modified CNG channel as claimed in claim 1, which comprises subunits of CNG channels from bovine and/or other organisms.

3. The genetically modified CNG channel as claimed in claim 1 or claim 2, which has a homooligomeric composition of identical subunits or a heterooligomeric composition of different subunits.

4. The genetically modified CNG channel as claimed in any of claims 1 to 3, which has at least one further genetic modification.

5. The genetically modified CNG channel as claimed in any of claims 1 to 4, which comprises chimeric subunits.

6. The genetically modified CNG channel as claimed in any of claims 1 to 5, wherein the amino acid corresponding to threonine T537 in the bovine α3 subunit has been replaced by methionine or valine.

7. A method for preparing CNG channels, which comprises replacing in the subunits the amino acid corresponding to threonine T537 in the bovine α3 subunit with a different amino acid other than serine.

8. The method as claimed in claim 7, which comprises replacing the amino acid corresponding to threonine T537 in the bovine α3 subunit with methionine or valine.

9. The use of the CNG channels as claimed in any of claims 1 to 6 for measuring the intracellular cAMP concentration.

10. The use of the CNG channels as claimed in any of claims 1 to 6 for determining the action of ligands, agonists and antagonists on G protein-coupled receptors and of activators and inhibitors on other proteins regulating the intracellular cAMP concentration.

11. A modified nucleic acid, which codes for subunits of the CNG channels as claimed in any of claims 1 to 6.

12. An expression vector, which comprises nucleic acids as claimed in claim 11.

13. A cell line, which comprises CNG channels as claimed in any of claims 1 to 6, modified nucleic acids as claimed in claim 11 or expression vectors as claimed in claim 12.

14. The cell line as claimed in claim 13, which is capable of expressing CNG channels as claimed in any of claims 1 to 6.

15. The cell line as claimed in either of claims 13 and 14, which is capable of heterologously coexpressing proteins regulating the intracellular cAMP concentration together with CNG channels as claimed in any of claims 1 to 6.

16. The cell line as claimed in any of claims 13 to 15, which is capable of heterologously coexpressing

G protein-coupled receptors, phosphodiesterases, adenylate cyclases together with a CNG channel as claimed in any of claims 1 to 6.

17. A method for preparing cell lines, which comprises transforming them with an expression vector as claimed in claim 12.

18. The method as claimed in claim 17, which comprises cloning the genes for the proteins into an expression vector as claimed in claim 12 and then transforming the cell lines.

19. The method as claimed in either of claims 17 and 18, which comprises using eukaryotic CHO, COS or SF9 cell lines as heterologous expression system.

20. The method as claimed in either of claims 17 and 18, which comprises using human embryonic kidney (HEK) 293 cell lines as heterologous expression systems.

21. A method for preparing cell lines as claimed in any of claims 13-16, which comprises expressing the proteins stably or transiently.

22. A method for preparing cell lines as claimed in either of claims 15 or 16, which comprises expressing one of the proteins stably and the other protein transiently.

23. The use of the cell lines as claimed in any of claims 13 to 16 for determining the intracellular Ca^{2+} concentration.

24. The use as claimed in claim 23, wherein the determination is carried out using fluorescence-optical methods.

25. The use as claimed in either of claims 23 and 24, wherein fluorescent Ca^{2+} indicators are used.

26. The use as claimed in claim 23, wherein the determination is carried out using luminescence-optical methods.

27. The use as claimed in either of claims 23 and 26, wherein luminescent apoaequorin or isoforms thereof are used.

28. The use as claimed in any of claims 23 to 27, wherein the Ca^{2+} concentration is determined in cuvette measuring devices or Ca^{2+} imaging apparatuses.

29. The use as claimed in any of claims 23 to 28, wherein the Ca^{2+} concentration is determined in fluorescence or luminescence readers.

30. The use as claimed in any of claims 23 to 29, wherein multiwell plates are used.

31. The use as claimed in any of claims 23 to 30, wherein adherent cells or cells in suspension are used.

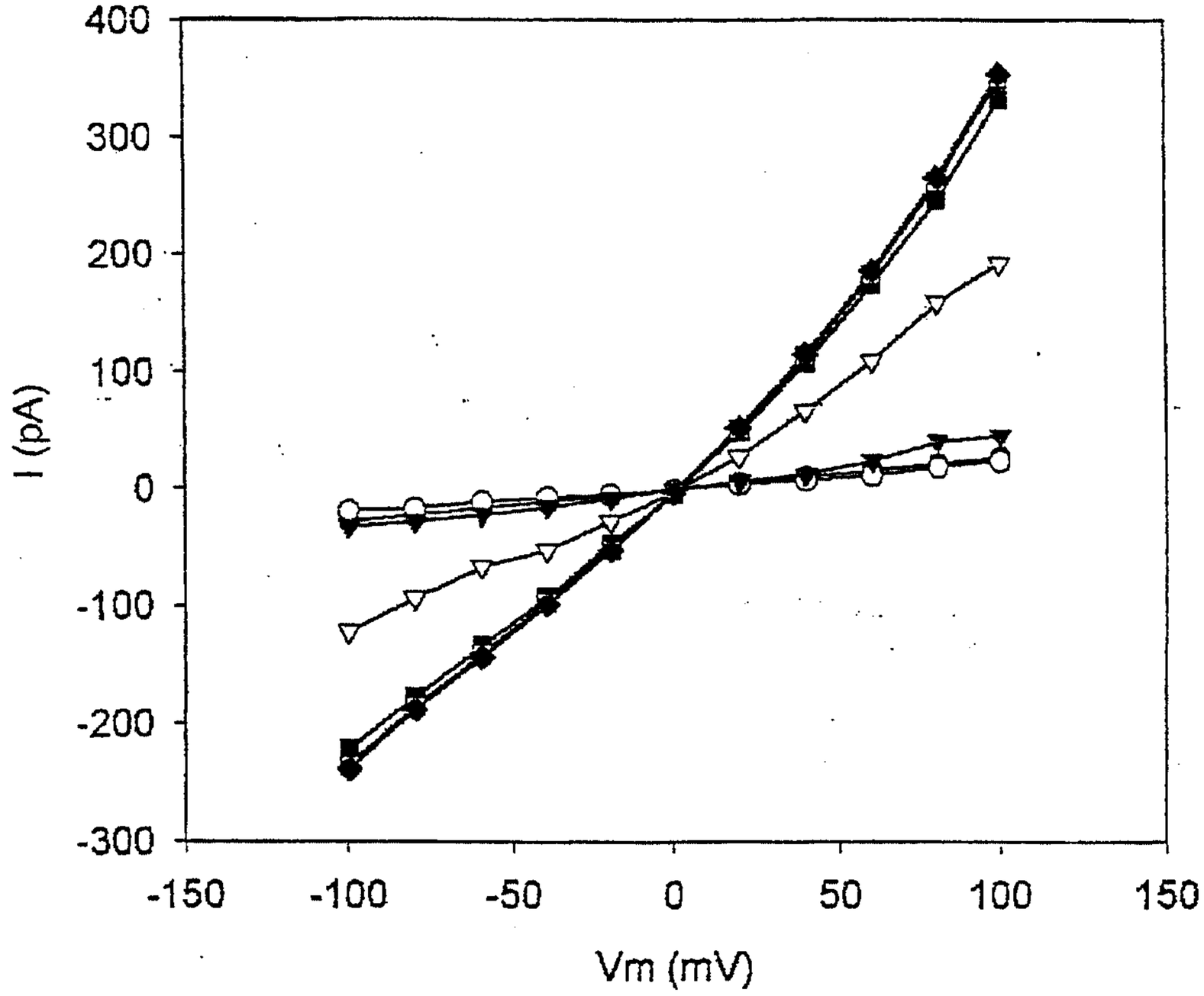
32. The use as claimed in any of claims 23 to 31 for characterizing pharmaceutically active compounds or pharmacological substances, which increase or reduce the intracellular cAMP concentration.

33. The use as claimed in any of claims 23 to 31 for characterizing GPCRs, adenylate cyclases, phosphodiesterases or other proteins regulating the cellular cAMP concentration.

34. The use as claimed in either of claims 32 and 33, wherein high throughput (HTS) or ultra-high throughput (UHTS) is employed.

35. The use as claimed in any of claims 23 to 34, wherein the measurements are carried out in real time.

Patent Agents
Smart & Biggar



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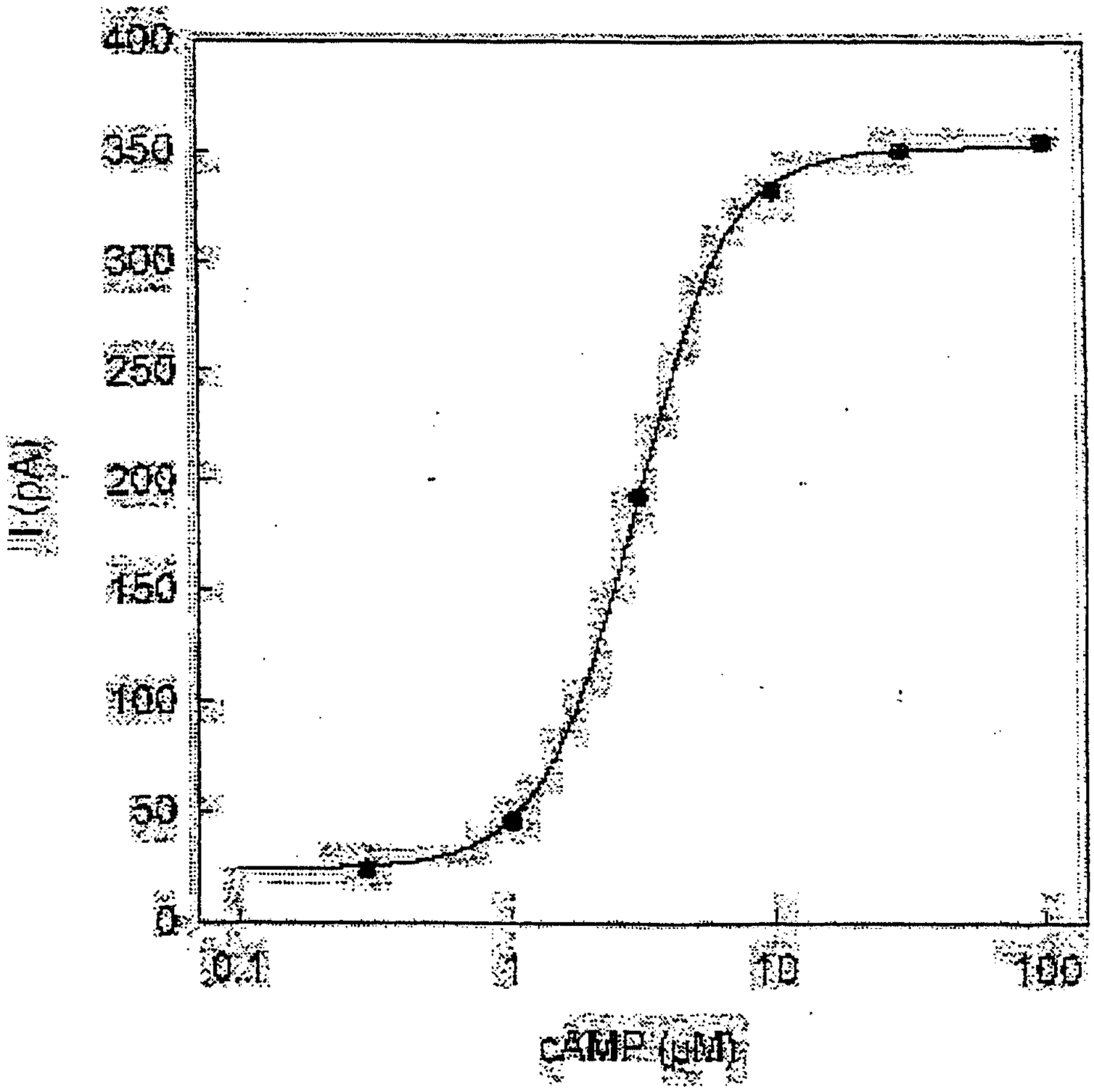

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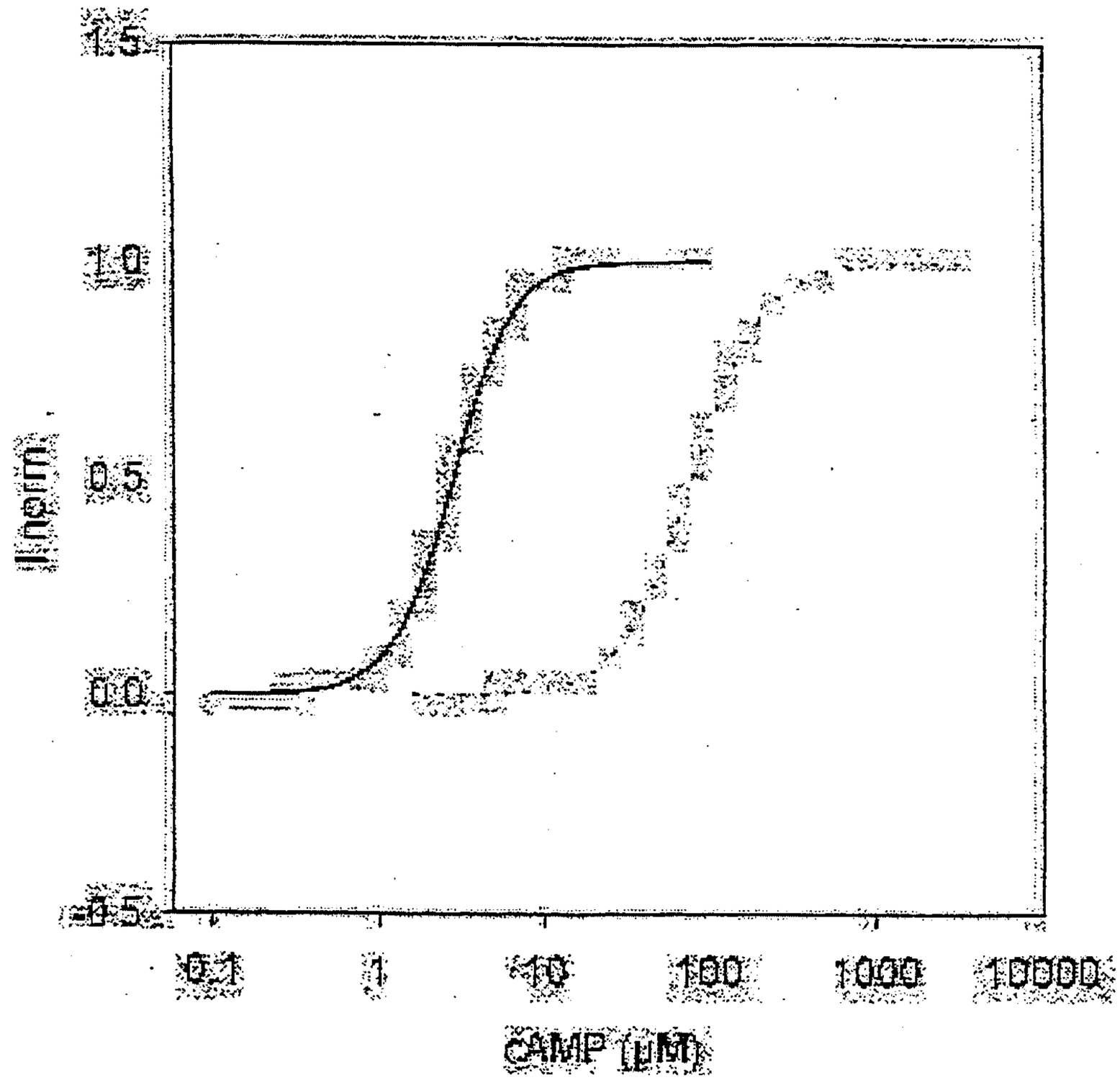

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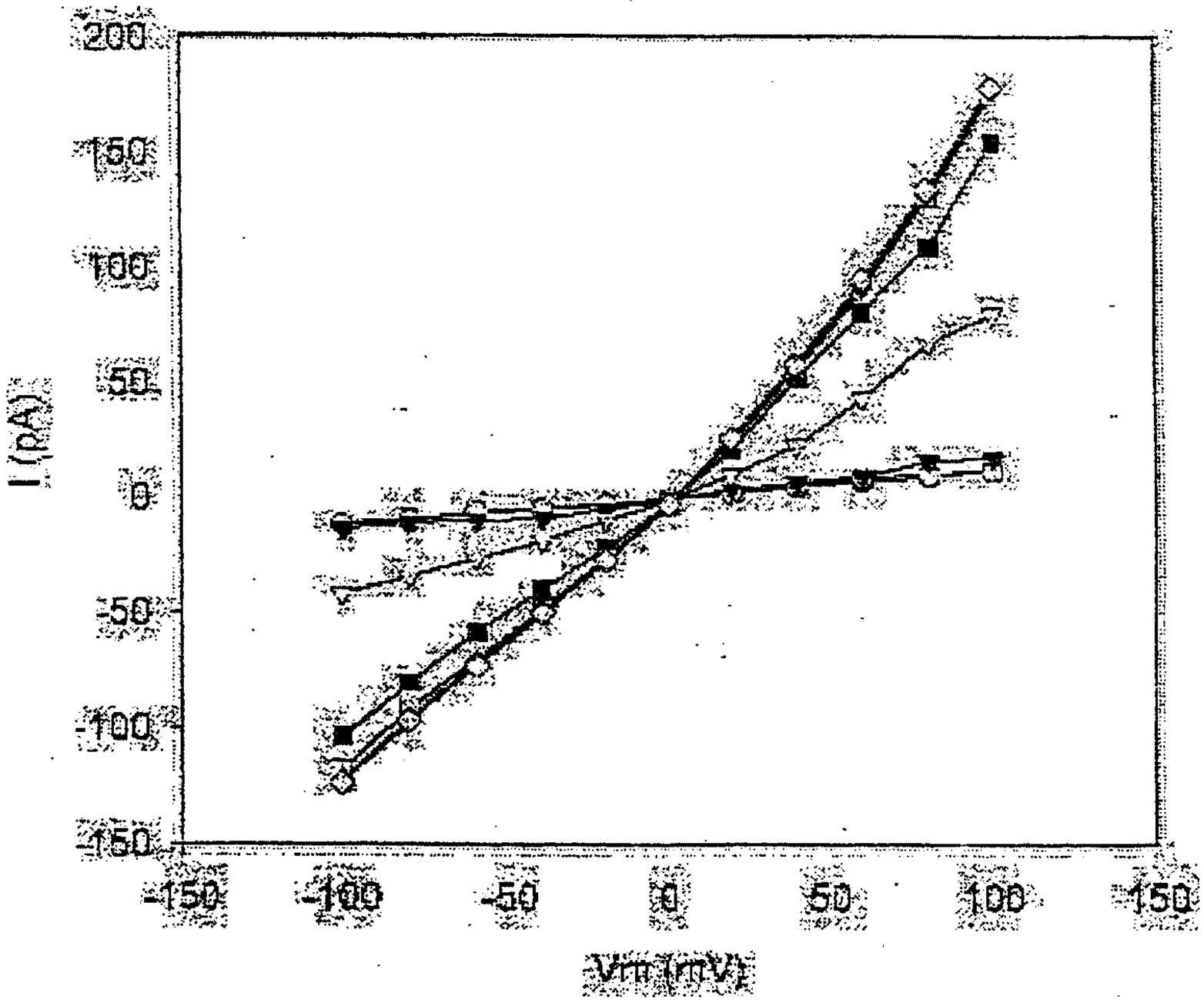

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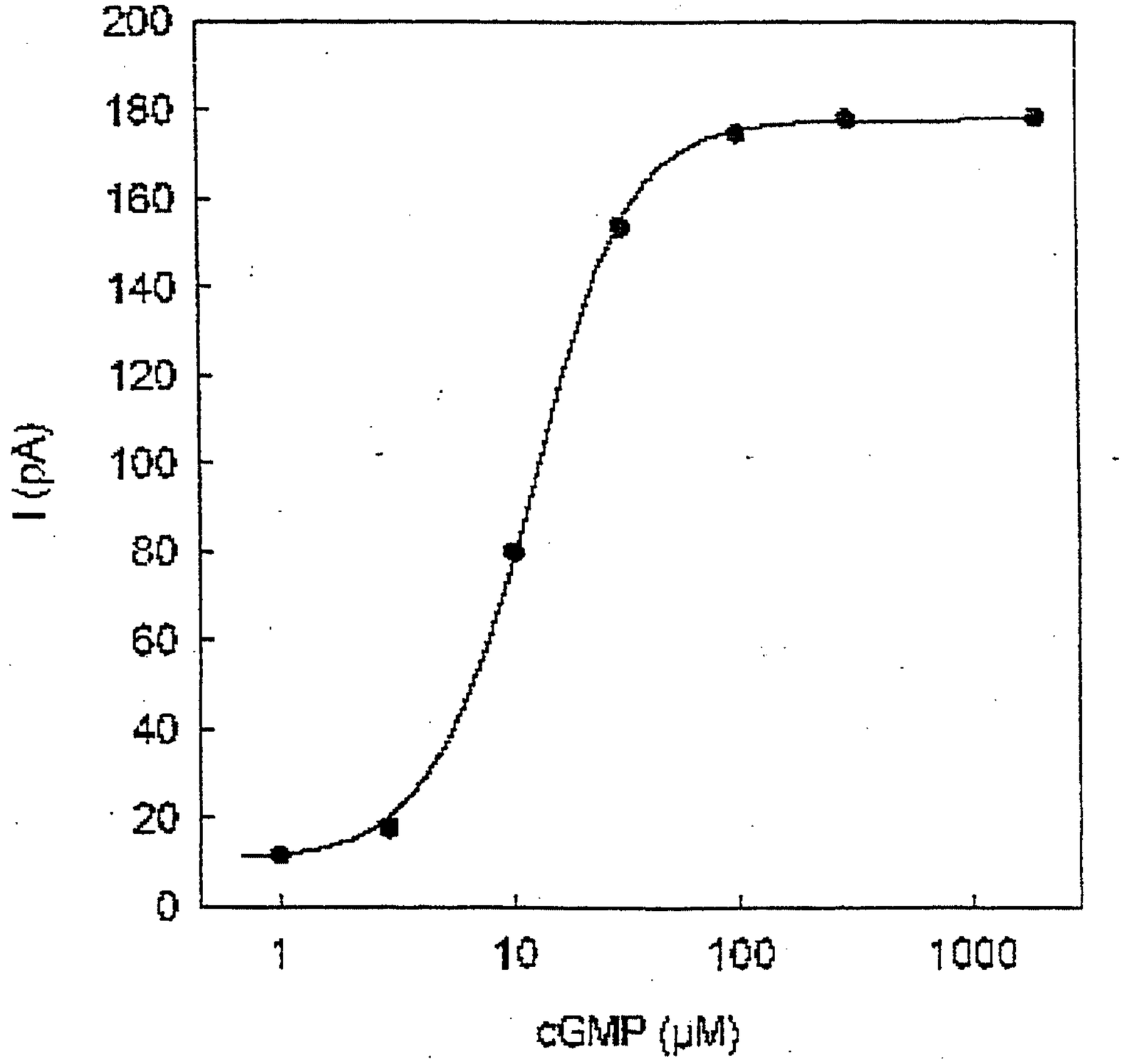

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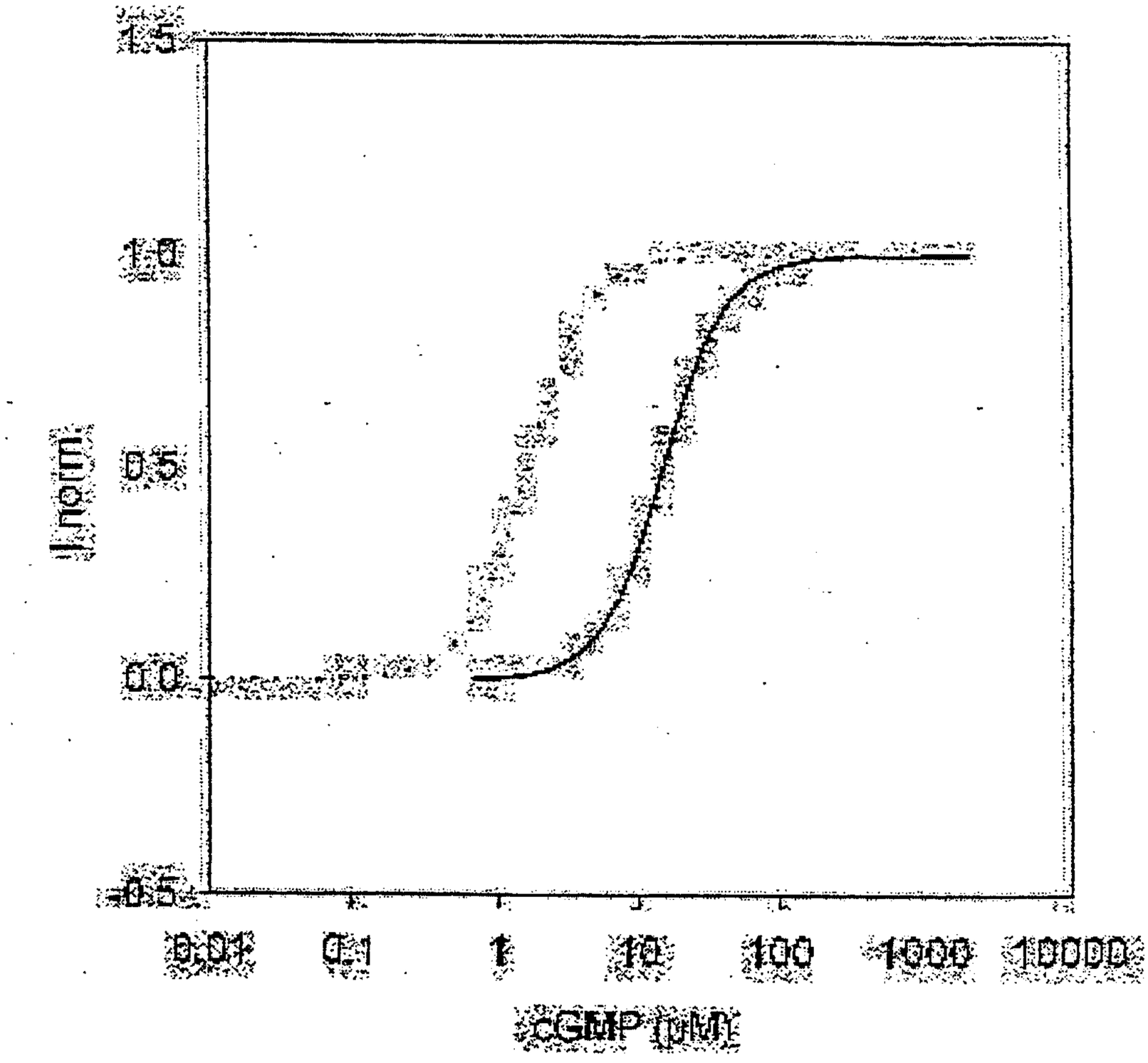

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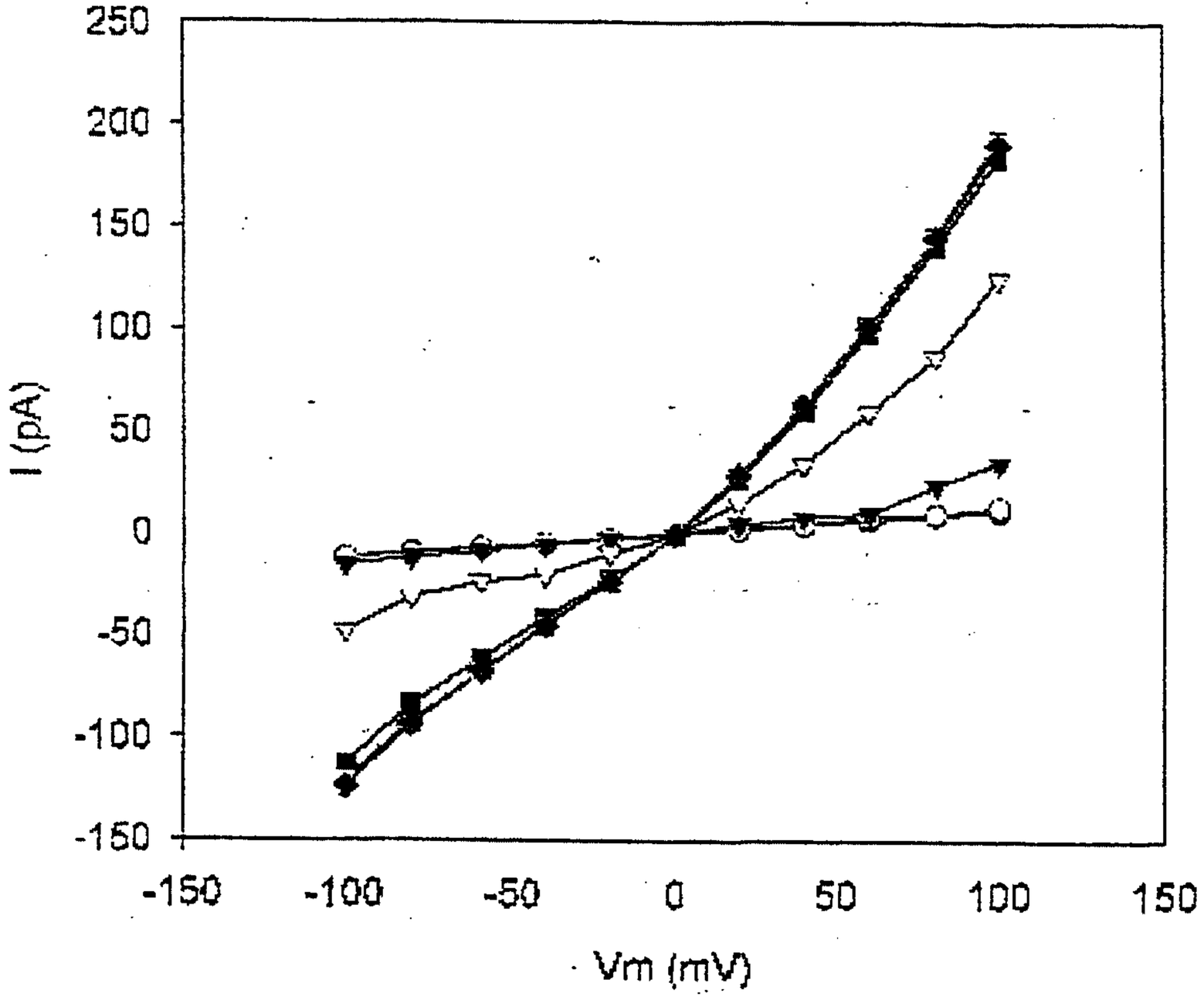

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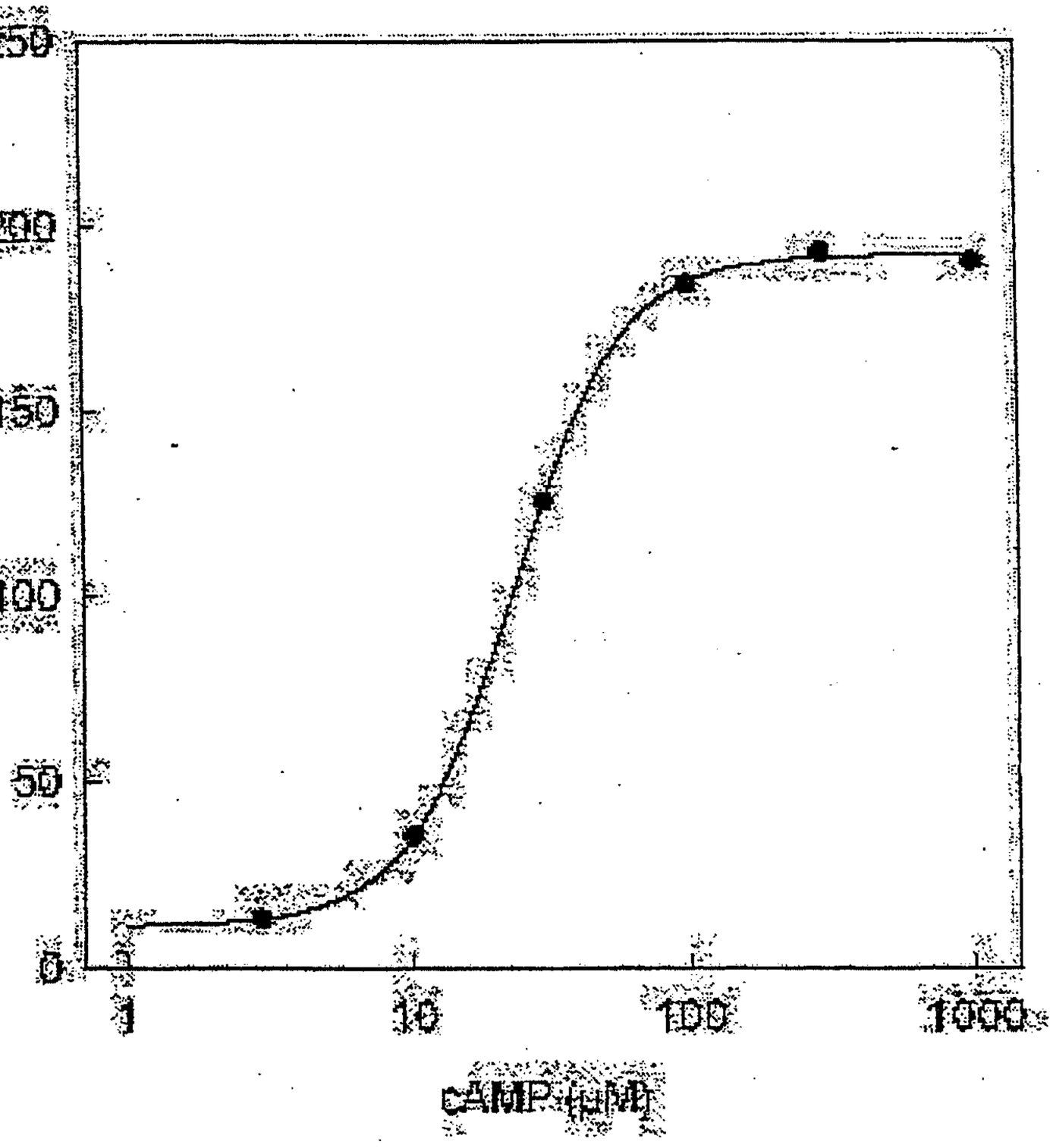

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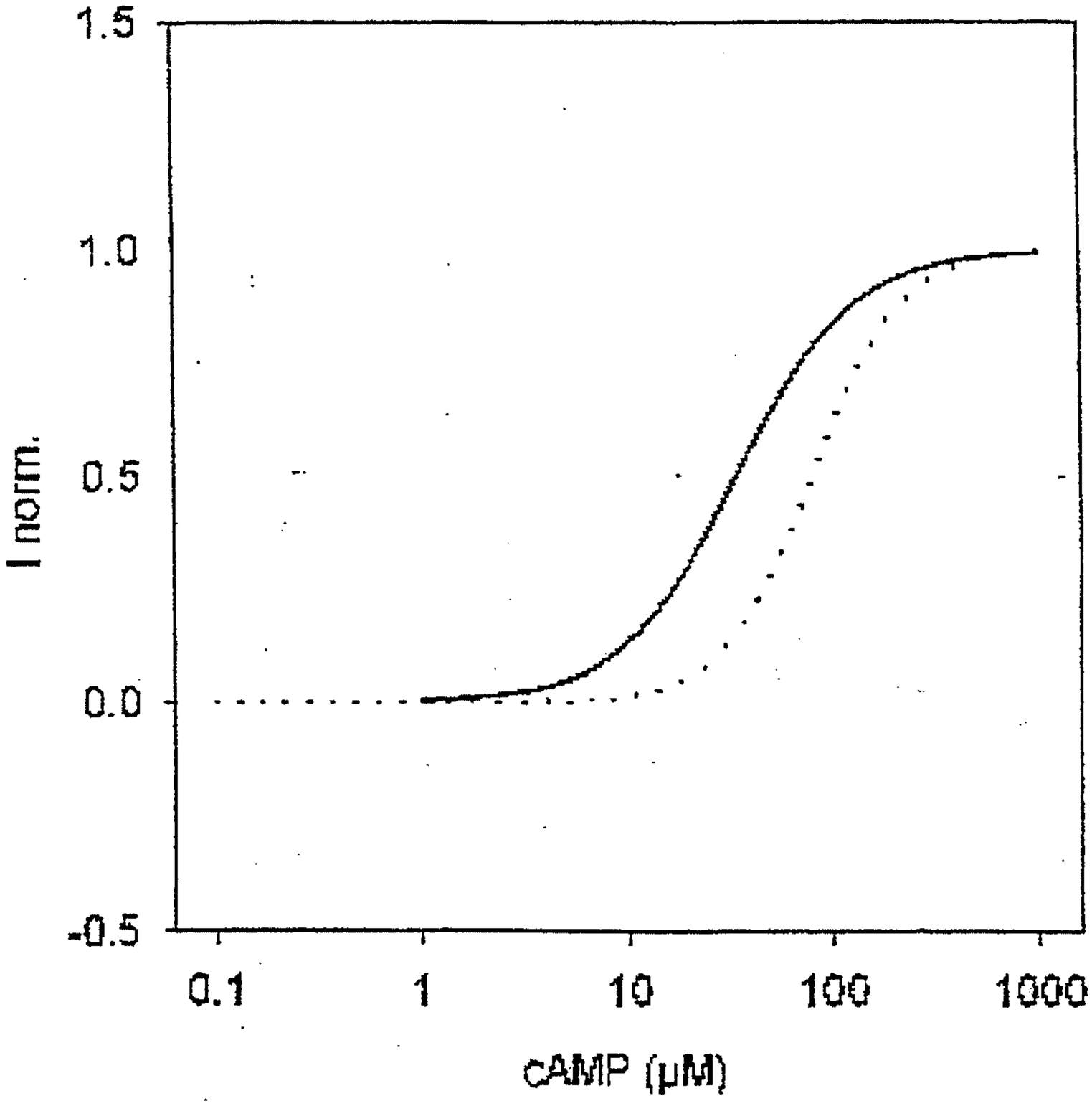

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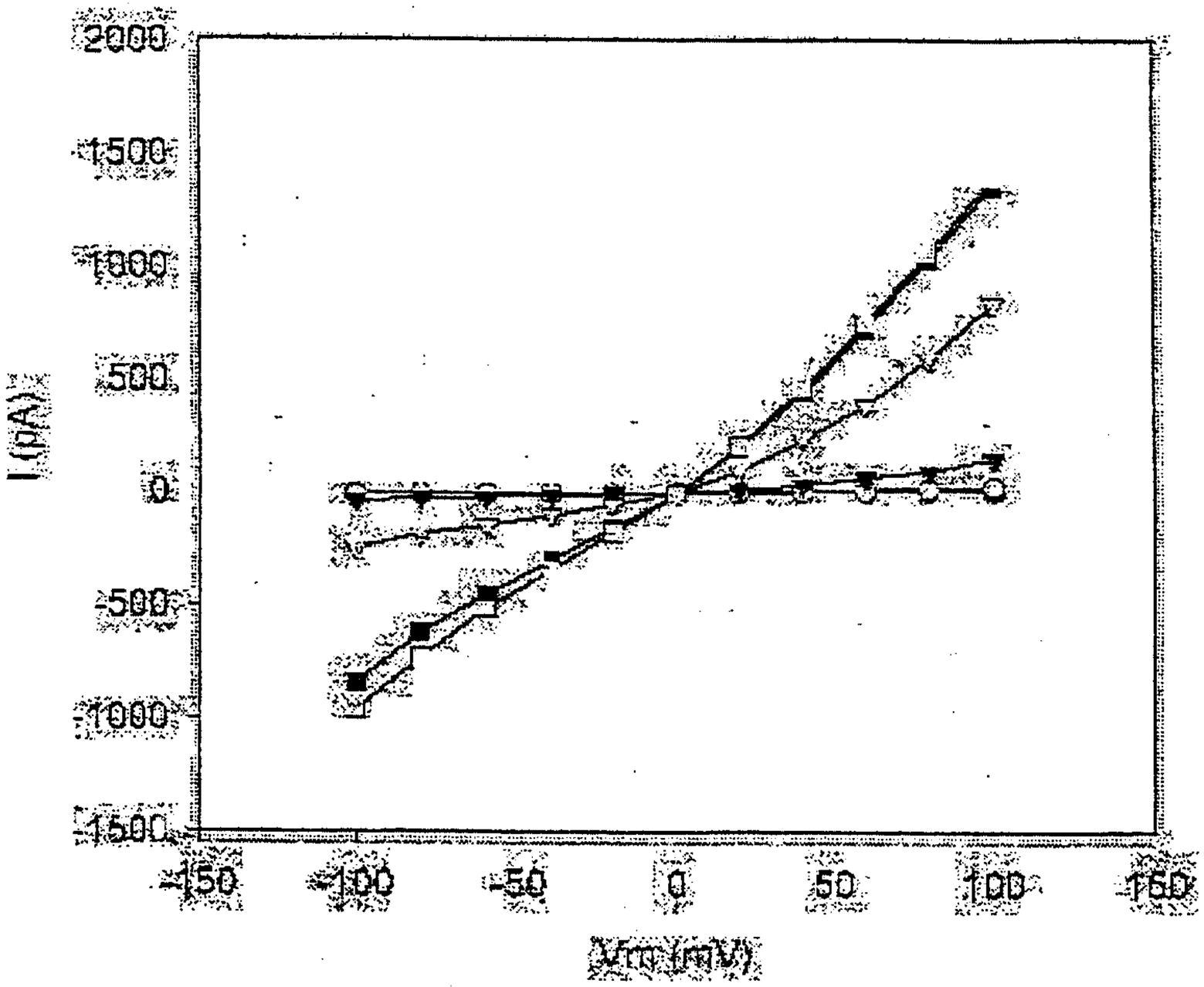
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Fig. 4A

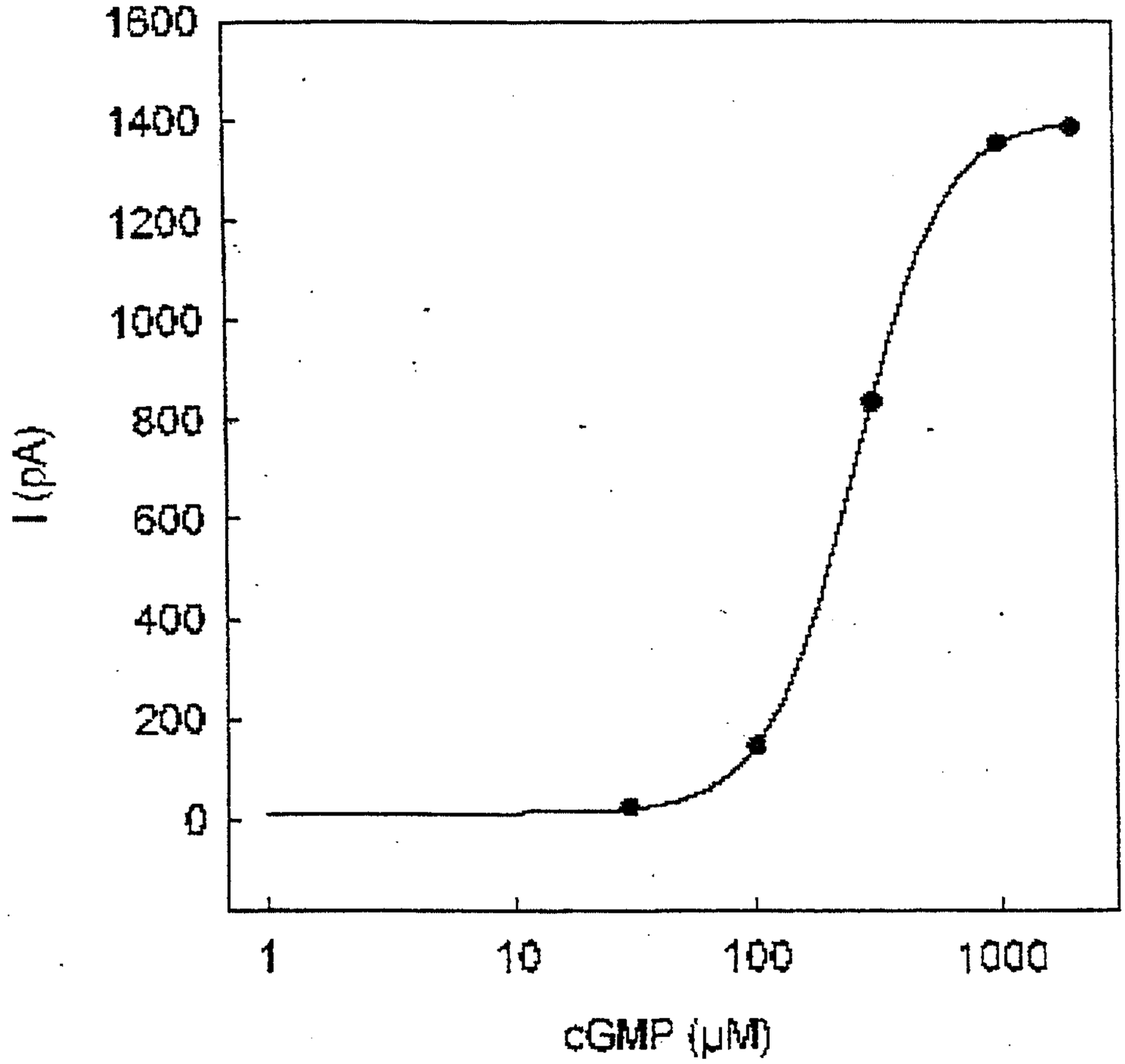

Fig. 4B

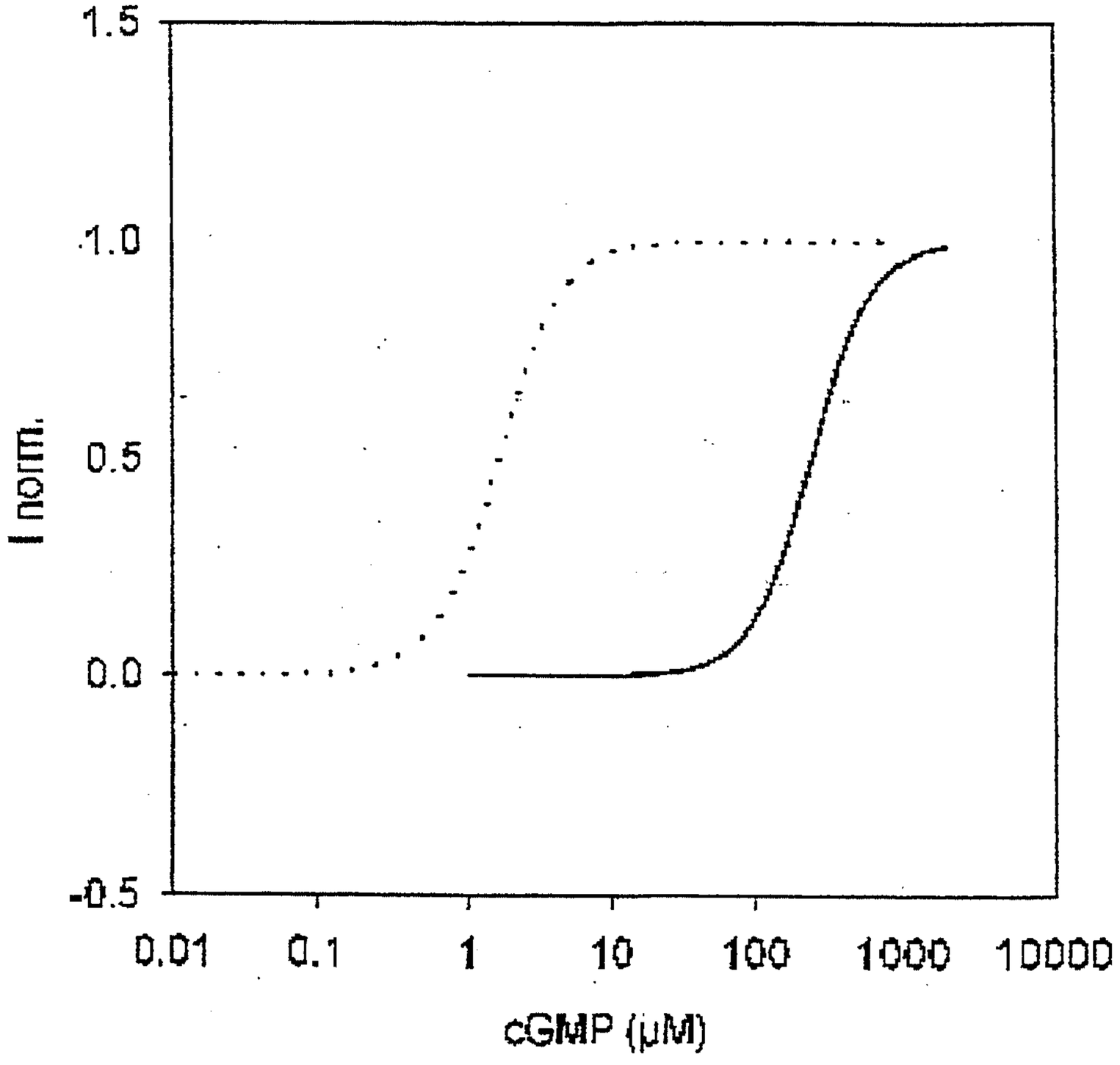

Fig. 4C

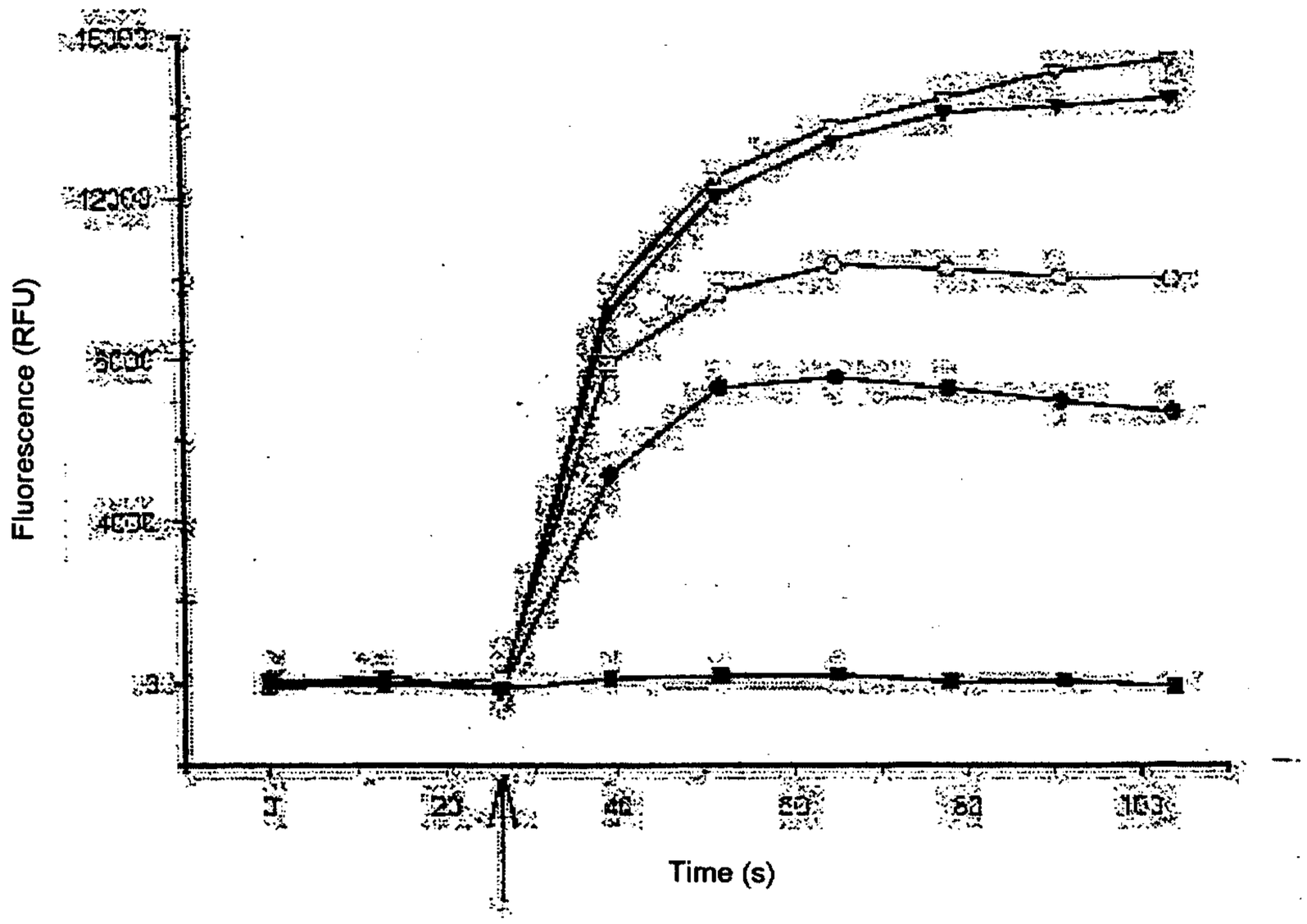

Fig. 5A

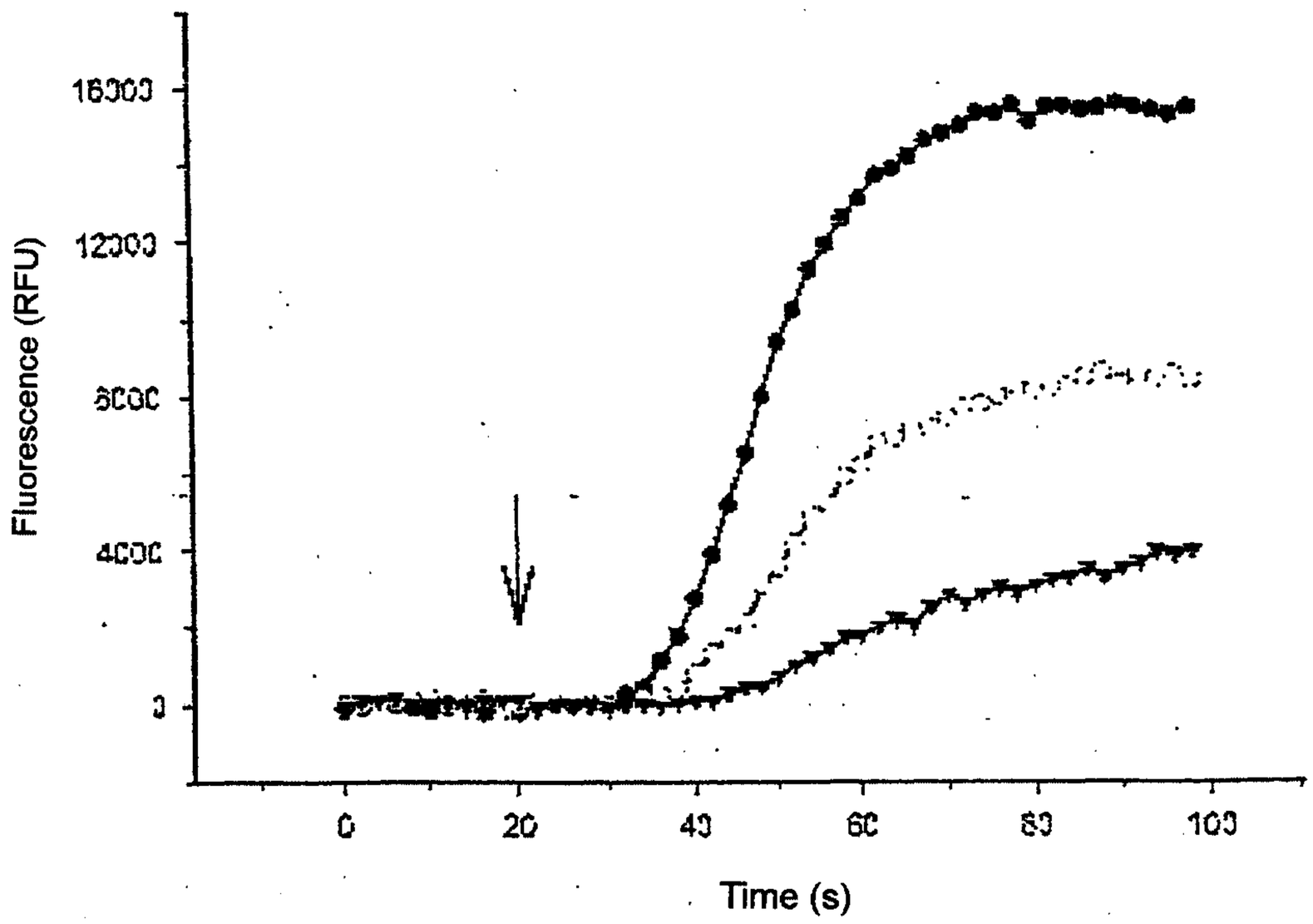
Fluorescence (RFU)
16000
12000
8000
4000
0
0
20
40
60
80
100
Time (s)

Fig. 5B

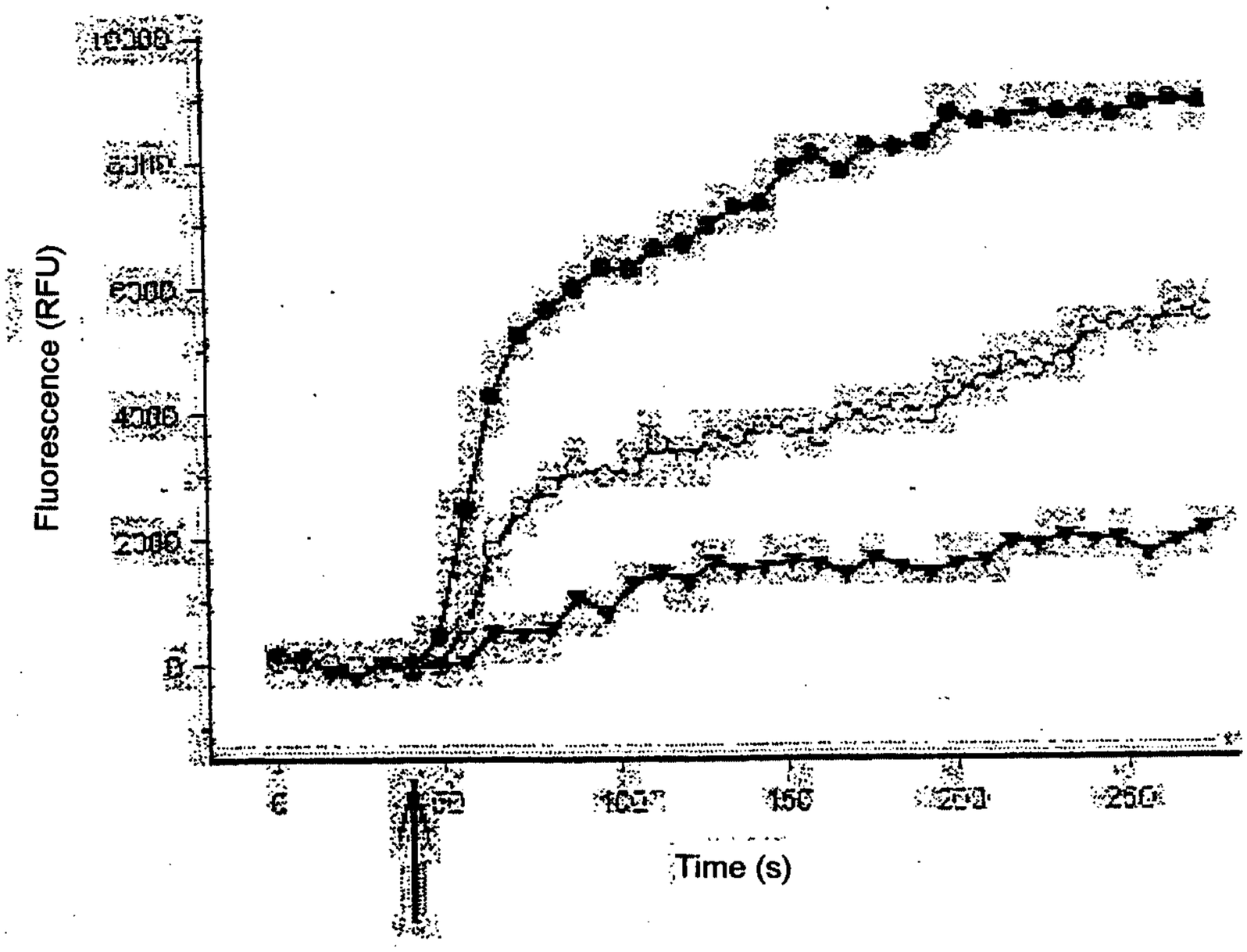

Fig. 5C

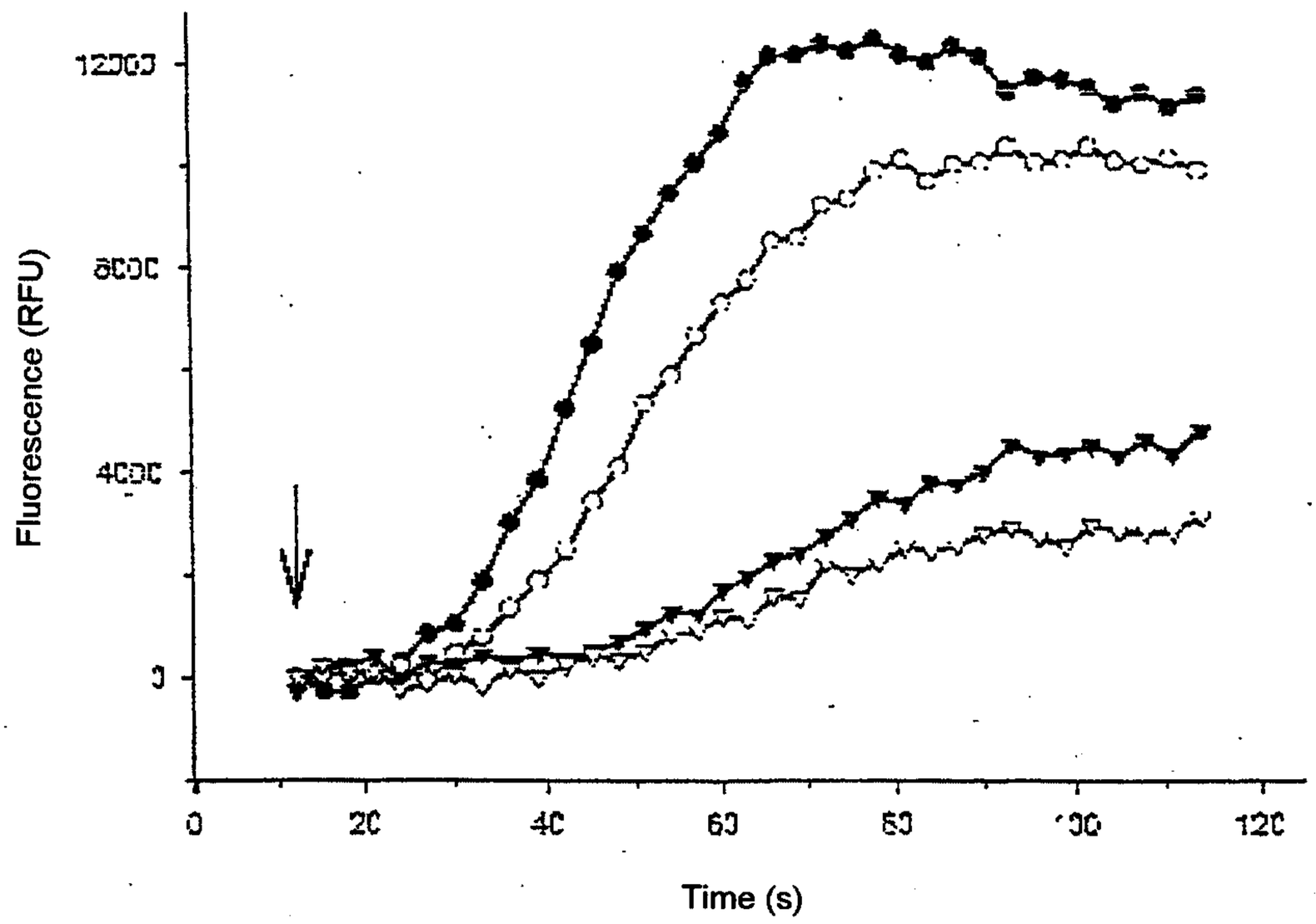

Fig. 5D

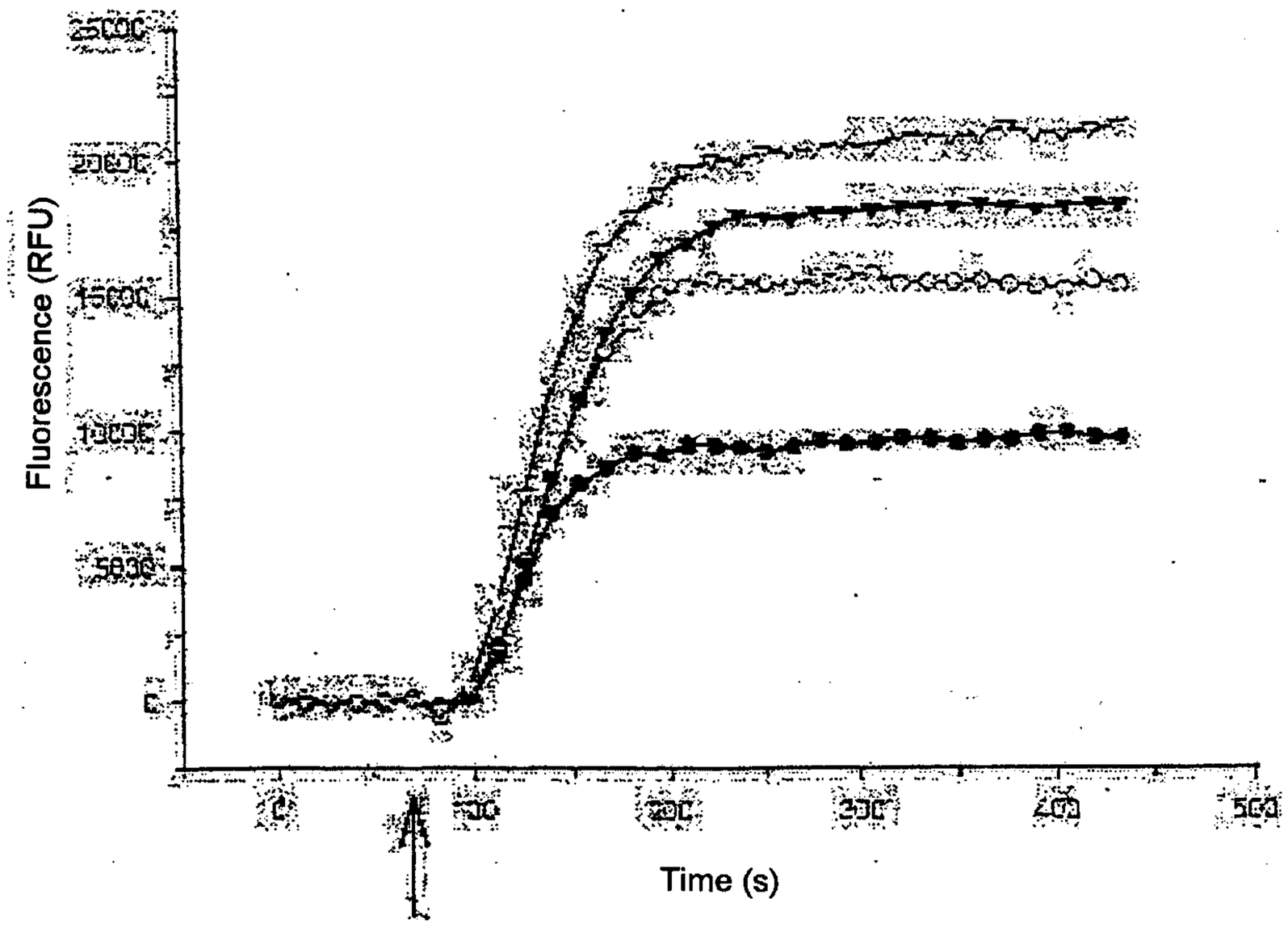
Fluorescence (RFU)
25000
20000
15000
10000
5000
0
0
10
20
30
40
50
Time (s)

Fig. 5E

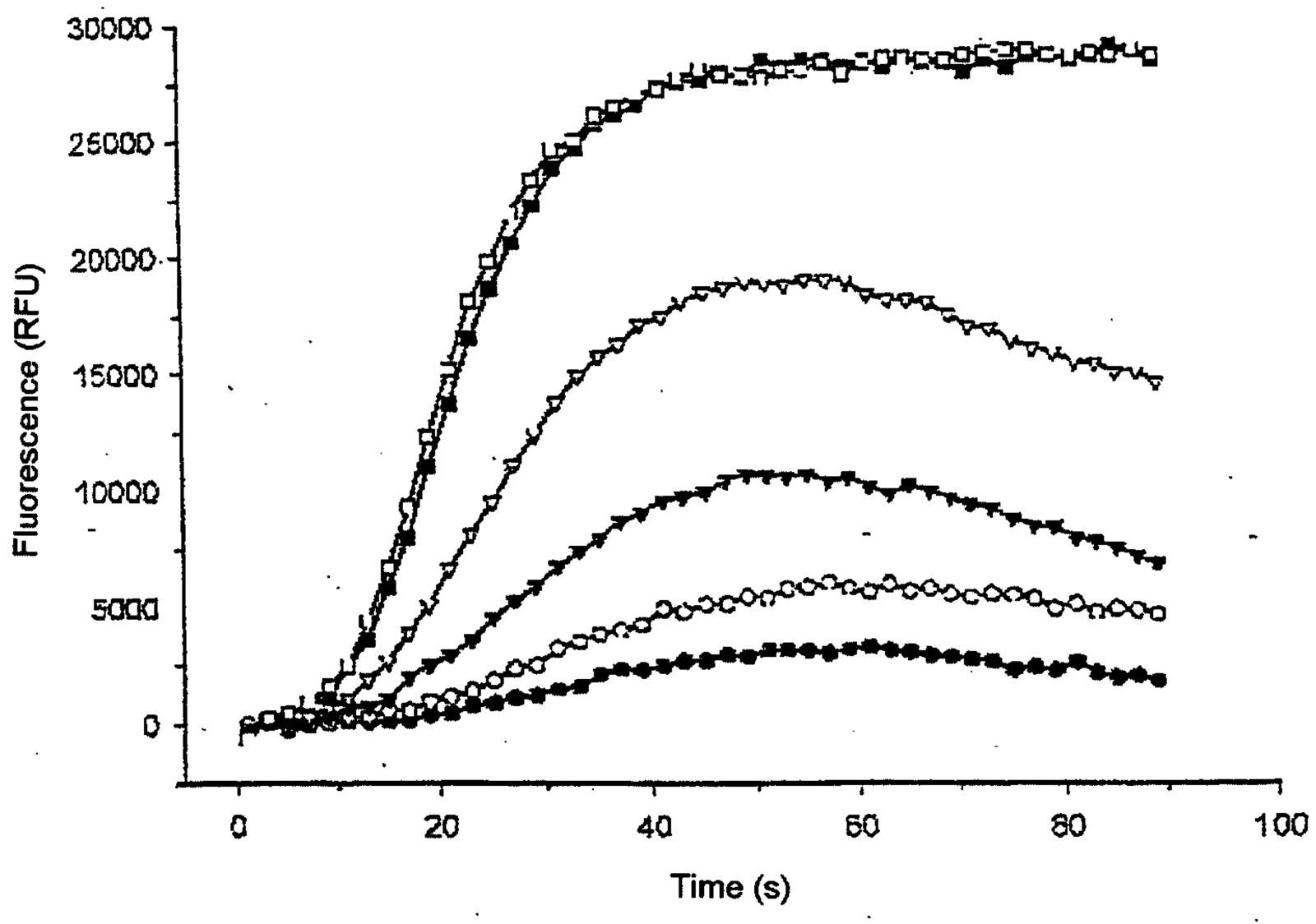
30000
25000
20000
15000
10000
5000
0
Fluorescence (RFU)
0
20
40
60
80
100
Time (s)

Fig. 5F(1)

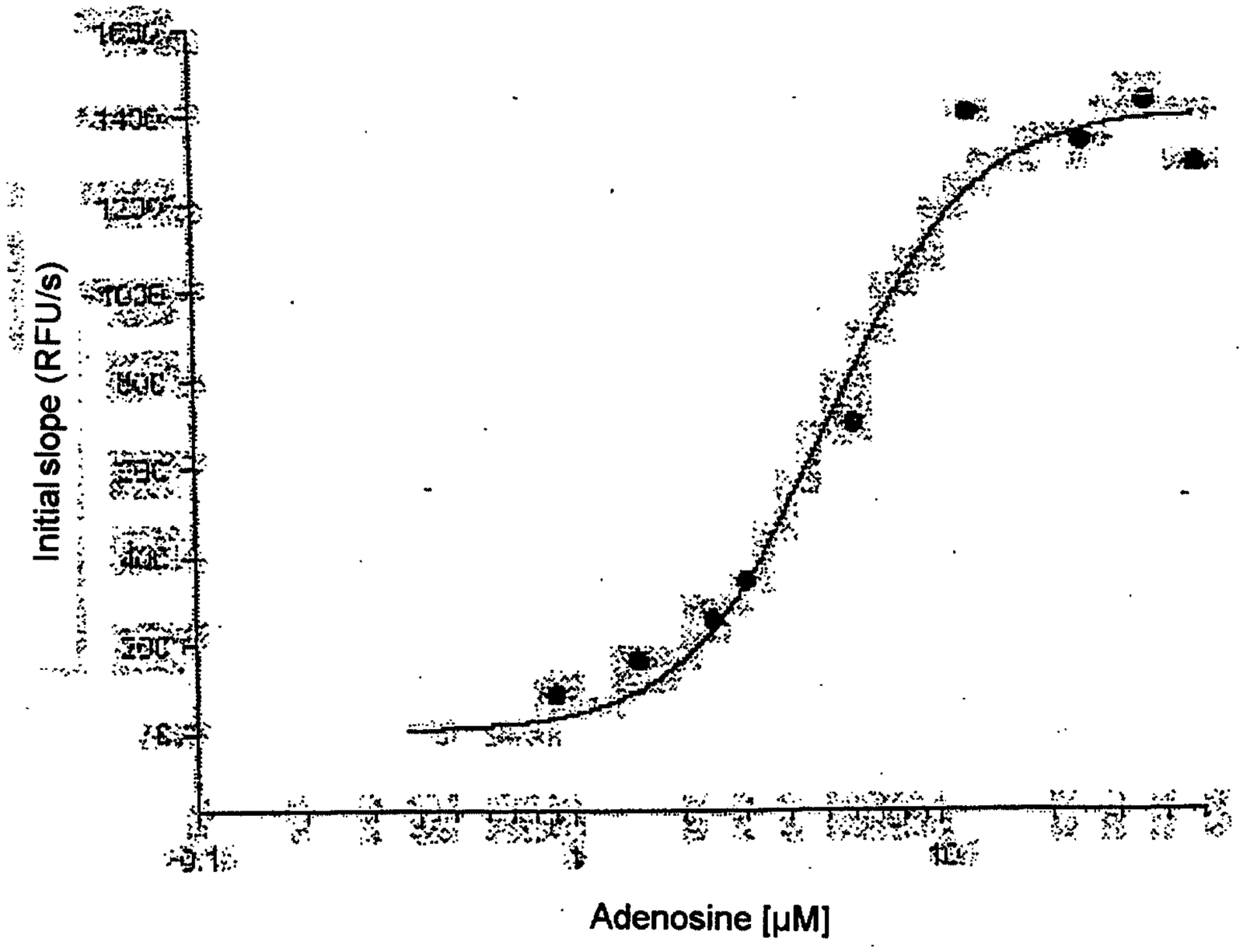

Fig. 5F(2)

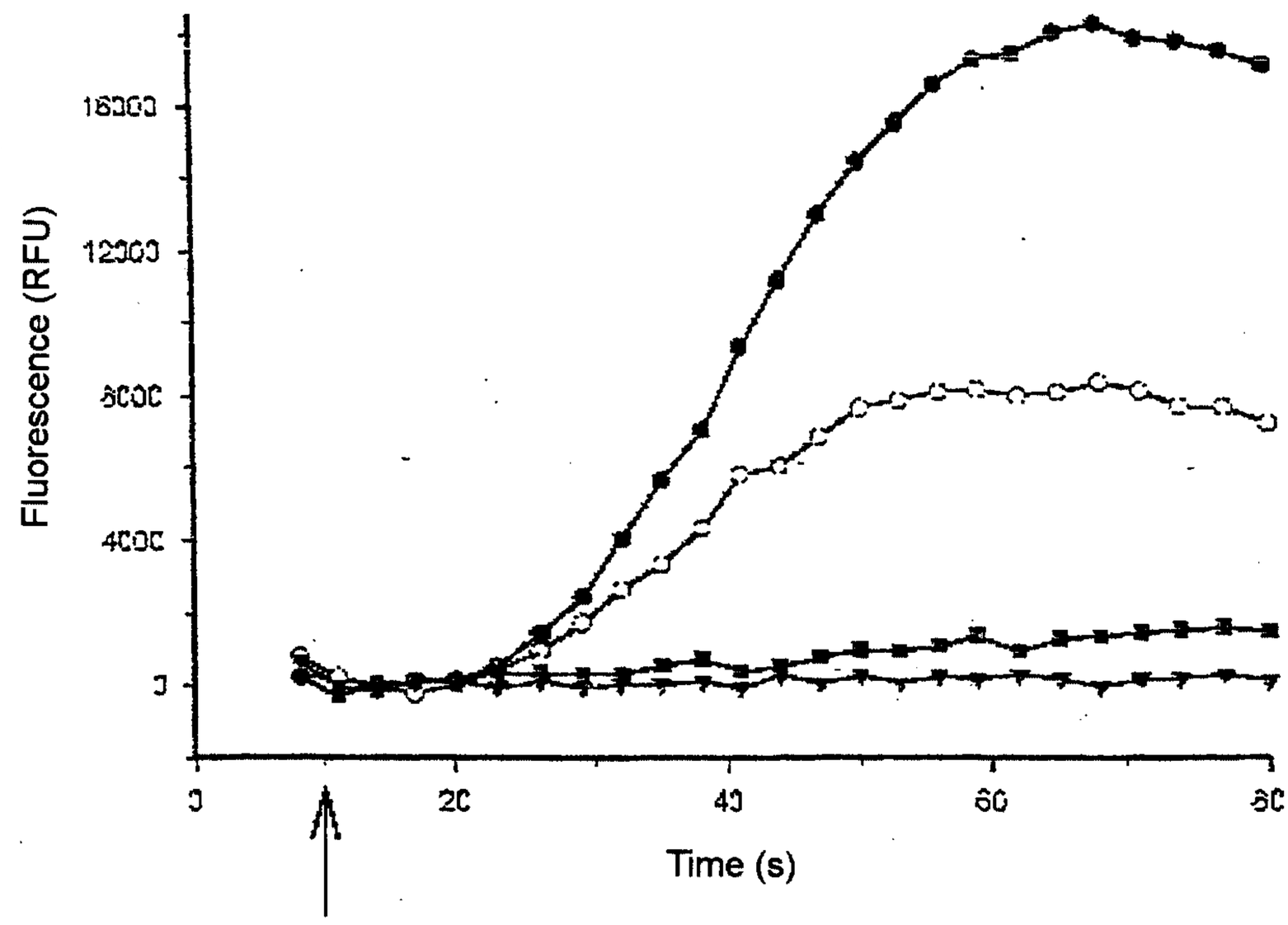

Fig.5G