

Expression of photoreceptor cyclic nucleotide-gated cation channel α subunit (CNGC α) in the liver and skeletal muscle

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Abstract Glucagon and β -adrenergic agents increase cAMP levels and stimulate Ca^{2+} influx in liver cells. There is no consensus as to the mechanism by which these hormones stimulate the influx of Ca^{2+} . Using mouse retinal rod CNGC α cDNA probes, we cloned rat liver and skeletal muscle, and human hepatic CNGC α subunit sequences showing 97–100% identity with the human rod channel. In order to assess channel activity, the effect of cyclic nucleotides on free intracellular Ca^{2+} levels of isolated hepatocytes was measured. Dibutyryl-cAMP was more effective in increasing free Ca^{2+} levels than dibutyryl-cGMP. These data indicate that the CNGC α subunit is expressed in the both liver and skeletal muscle possibly mediating hormonal effects on ion fluxes.

Key words: Cyclic nucleotide-gated channel; Liver; Skeletal muscle; Photoreceptor; Intracellular calcium

1. Introduction

Cyclic nucleotide-gated (CNG) cation channels are directly gated by cGMP and, to a lesser extent, by cAMP [1,2]. These channels, first described in the retinal rod photoreceptor, are heteromeric [3,4] and consist of at least two subunits (α and β). The α subunit is a 63 kDa polypeptide, containing cGMP binding sites, and six putative transmembrane regions [5,6]. When expressed in heterologous systems, the α subunit forms an active channel which is insensitive to the Ca^{2+} blocker *L-cis*-diltiazem. The β subunit is a 240 kDa polypeptide, consisting of a glutamic acid rich N-terminal half (GARP), and a C-terminal half containing cyclic nucleotide binding site and transmembrane regions [7]. Co-expression of α and β subunits establishes a functional channel showing all features of the native channel. Another light-sensitive organ, the pineal gland, also possesses a cGMP activated cation channel [8]. A cAMP activated cation channel is present in the olfactory system, a channel which is likely to mediate olfactory transduction [9,10]. In this channel, the cAMP sensitivity is conferred by the β -subunit [11,12]. The presence of cyclic nucleotide gated channels, however, is not restricted to sensory organs. More recently, the presence of such channels was demonstrated in testis, kidney and heart [13,14]. Similar channels were identified also in other tissues such as pancreas, colon, adrenal gland, and ovary cells [15].

In the liver, early metabolic studies followed up with measurements of ion fluxes indicated the possibility that cyclic nucleotide-gated channels might be involved in the responses to cAMP synthesis. It was the consequence of studies aimed at elucidating the mechanisms of hormone action in the liver that cAMP was discovered [16]. First cAMP and later cGMP were shown to simulate the effects of hormones, namely glucose output, lactate uptake, urea production and the induction of tyrosine amino transferase [17–19]. In early studies, it was also established that glucagon, catecholamines, cAMP and cGMP administration is followed by an immediate uptake of K^+ and Na^+ into the liver followed by K^+ release and membrane hyperpolarization. A release of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) seemed to start simultaneously or slightly after the beginning of K^+ uptake [20,21]. These hormones also stimulated the influx of Ca^{2+} into liver cells [22–24]. These changes in ion distribution were shown to be essential for the manifestation of the hormonal effects on metabolism [25]. However, the ion channel responsible for these fluxes has yet to be identified. This study was undertaken to explore the presence of cyclic nucleotide gated channels in the liver.

2. Materials and methods

2.1. Northern blot analysis

A multiple tissue blot (Clontech) was probed with a 2 kb fragment (subclone MCC13) [6] of the mouse rod CNGC α subunit. Each lane of the blot contained approximately 2 μg of poly(A) RNA. Hybridization conditions followed the protocol suggested by Clontech Laboratories.

2.2. Polymerase chain reaction (PCR)

mRNA from liver and skeletal muscle of male Sprague-Dawley rats weighing 130–200 g was isolated using FastTrack (Invitrogen Corp., San Diego, CA) and reverse transcribed following the instructions of the reverse transcription kit. PCR reactions were performed in 25–60 μl volumes containing 2/25 volume of diluted cDNA and 10–20 pmol of each primer in an MJ Research PTC-100 thermal cycler for 40 cycles as described previously [6]. The sequences of primers MCC-F, RH-1, and W73 are given in [6]. The PCR products were resolved on a 1% agarose gel, extracted from the gel, cloned into PCRII vector (Invitrogen) and sequenced at the University of Texas-Houston Health Science Center DNA sequencing core facility employing a ABI PRISM 377 DNA sequencer.

2.3. Screening of human cDNA library

Approximately 1 000 000 plaque forming units of a λ Uni-ZAP XR library (Stratagene, La Jolla, CA.) were screened with the PCR amplified fragment which we obtained using liver mRNA and the MCCF/RH-1 primers [6]. The fragment was labeled with [^{32}P]dATP by nick-translation. Ten positive clones were identified, plaque-purified and excised into pBluescript phagemid following the manufacturer's protocol.

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2.4. Preparation of subcellular fractions

Rat liver plasma membranes were obtained as described [26]. Rat retinal homogenate was prepared by homogenization in 10 mM Tris-HCl, pH 7.5, 0.1% NP-40, 1 mM EDTA, 1 mM PMSF, and 1 mg/ml inhibition cocktail (equal amounts of leupeptin, antipain, and pepstatin A). The homogenate was spun for 15 min at 4°C in a microcentrifuge. An aliquot of the pellet was resuspended in SDS sample buffer.

2.5. SDS-PAGE and Western blot analysis

SDS-PAGE [27] was performed in 10% separating gels. Subsequently, the gels were stained with Coomassie blue. For Western blot analysis, SDS gels were electrophoretically transferred to nitrocellulose filters. For immunostaining, the blots were incubated for 1 hour at room temperature or for 12 hours at 4°C in Tween 20-Tris buffered saline (TTBS, 0.05% Tween 20 in TBS) with 5% nonfat dry milk and then incubated with primary antibodies. The CNGC α -specific monoclonal antibodies (PMc 1D1, PMc 2G11 and PMc 1F6) were diluted to 1:20 in TTBS with 5% nonfat dry milk. After washing three times with TTBS, the blots were incubated with secondary antibodies (anti-mouse IgG, horseradish peroxidase conjugate, 1:50 000 dilution in TTBS) for 1 h at room temperature, washed twice with TTBS and once with TBS, and developed using the ECL chemiluminescence system (Amersham).

2.6. Measurement of $[Ca^{2+}]_i$ with fura-2 AM

Hepatocytes were isolated as described previously [28,29]. $[Ca^{2+}]_i$ was measured employing the fluorescence of fura-2 acetoxymethyl-ester (fura-2 AM) [29]. Briefly, cells were loaded with fura-2 AM, sedimented at $500\times g$ for 1 min and resuspended in a buffer containing 1.25 mM probenecid in order to block the export of fura-2 AM from the cells [30]. All subsequent solutions for cell suspensions contained probenecid (1.25 mM). The fluorescence of fura-2 AM loaded cells was monitored at 37°C in an LS-5 Perkin-Elmer fluorescence spectrophotometer. The instrument was controlled by a personal computer with the help of a program developed in-house by Perkin-Elmer personnel (Dean Brown). The fluorescence was excited alternatively at 340 and 380 nm, the emission was recorded at 510 nm, and the autofluorescence corrected ratio and $[Ca^{2+}]_i$ were calculated by a standard formula [31]. Cyclic nucleotide homologues, $N^6,2'$ -*O*-dibutyryl adenosine 3':5'-cyclic monophosphate, and $N,2'$ -*O*-dibutyryl guanosine 3':5'-cyclic monophosphate (Sigma Chemical Co., St. Louis, MO) were added as indicated in Fig. 5. Each experiment was repeated 4–8 times with similar results.

3. Results

3.1. Northern blot

In order to test for the presence of CNGC α in non-sensory rat tissues, we probed a multiple tissue RNA blot with mouse CNGC α (Fig. 1). The DNA probe hybridized to the liver RNA as indicated by the presence of several bands ranging from 2.5–9.0 kb in size. Distinct hybridization patterns were obtained with RNA samples from all the organs with the exception of the brain. Negative results with brain poly(A) RNA were reported also by another group [13]. In the spleen, heart and skeletal muscle, the major mRNA species detected by mouse CNGC α is approximately 7.5 kb. In lung, skeletal muscle and testis, the several species ranging from 2.5–9 kb are observed. The strongest hybridization was observed with RNA from the liver and lung. These results suggest that a mRNA related to CNGC α is present in the liver and also in many other organs.

3.2. RT-PCR of liver and skeletal muscle RNA

Based on the known sequence of the rod channel [6], specific primers which would amplify a portion of the cyclic nucleotide binding site of CNGC α were employed using either liver or skeletal muscle first strand cDNA (Fig. 2). In Fig. 2A, the results obtained with three sets of primers using hepatic

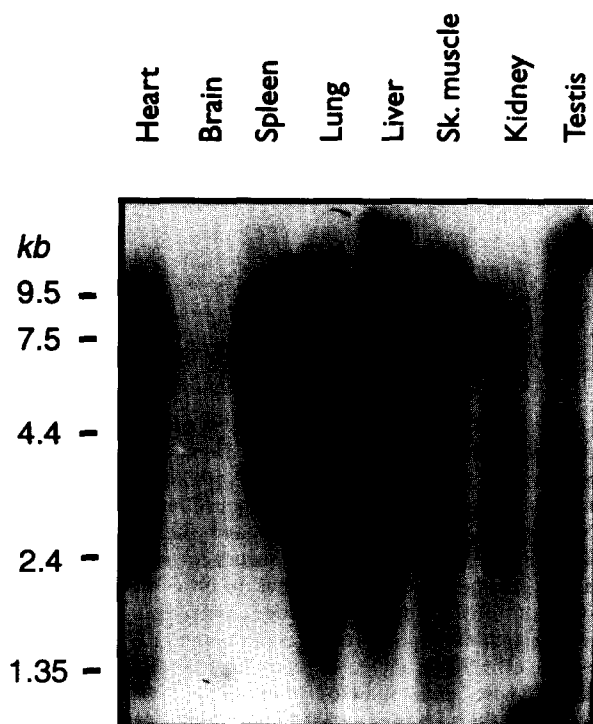


Fig. 1. Northern blot analysis of RNA from different rat tissues. Approximately 2 μ g of poly(A) RNA from the indicated rat organs were loaded in each lane. The blot was probed with a 2 kb cDNA fragment encoding the mouse rod CNGC α subunit. The size standards are indicated on the left.

first strand cDNA are shown. Each set of primers gave positive results and generated a DNA fragment of the expected size. In Fig. 2B, the results obtained employing cDNA derived from skeletal muscle and one set of primers in different buffers is shown. As in the liver, the expected size product was obtained. No fragments were observed in negative controls. These results confirmed the presence of mRNA for CNGC α both in the liver and in skeletal muscle.

3.3. Sequence analysis

The PCR products obtained with primers MCC-F and Rh-1 from rat liver and skeletal muscle (not shown) were sequenced and the predicted amino acid sequences aligned with the mouse CNGC α sequence (Fig. 3). In addition, clones were isolated from a human liver cDNA library and sequenced. The human liver cDNA sequences represented partial clones and were identical to human CNGC α subunit sequences reported earlier [6]. The rat liver and skeletal muscle CNGC α sequences were identical to each other and nearly identical to the mouse rod CNGC α sequence [6], and a mouse kidney inner medullary collecting duct CNGC α [14]. They also bear strong similarities to the cAMP-gated channel of the olfactory channel [9]. Deduced amino acid sequences of the clones obtained from the human liver library are identical to the human rod CNGC α sequence [6]. Thus, the results show that the CNGC α gene is expressed in human and rat liver, and the rat skeletal muscle.

3.4. Western blot

In order to test whether the channel protein can be detected, western blot analysis was carried out with monoclonal

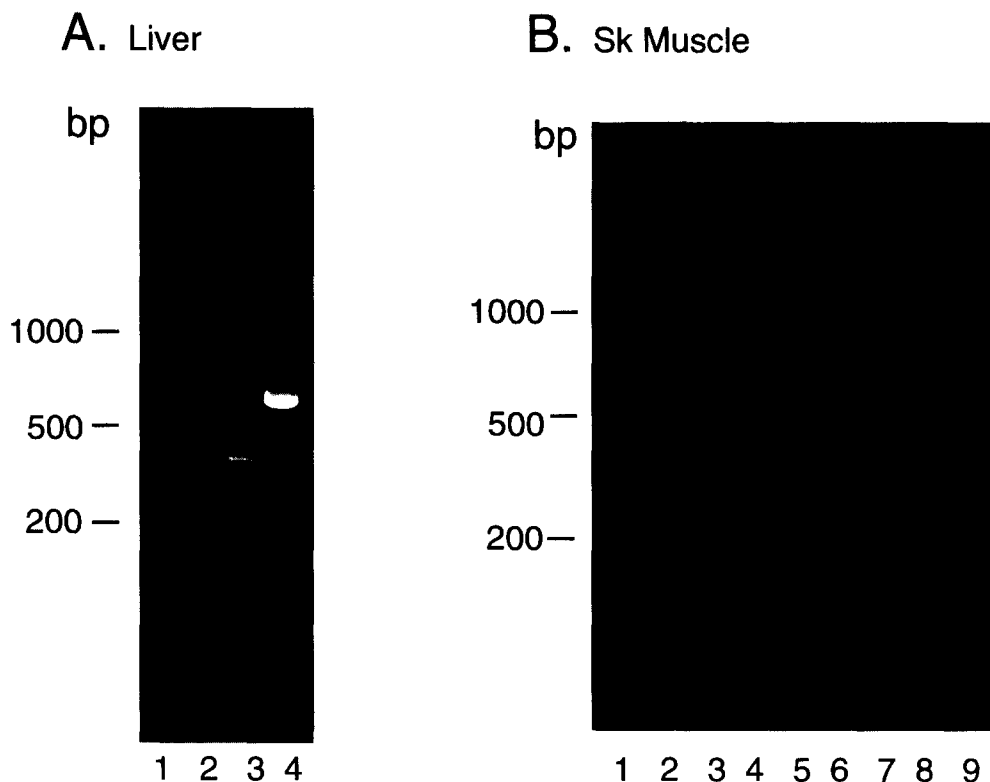


Fig. 2. In vitro amplification of liver, skeletal muscle and rod channel cGMP-gated cation channel. PCR products obtained from reverse transcribed rat liver (A) and skeletal muscle (B) mRNAs were resolved on a 1.5% agarose gel with 0.5 $\mu\text{g/ml}$ ethidium bromide. (A) Lanes: 1: negative control (absence of cDNA template); 2: liver cDNA with primers MCCF/MCCR; 3: liver cDNA with primers MCCF/W73; and 4: liver cDNA with primers MCCF/RH-1. (B) Lane 1: positive control MCC13 cDNA; 2: negative control; 3–9: skeletal muscle samples with different reaction buffers from PCR Optimizer Kit, primers MCCF/MCCR.

antibodies raised against C-terminal peptides of the rod channel (Fig. 4). The interaction of the three antibodies (PMc 1D1; PMc 2G11; and PMc 1F6) with rat retina and liver plasma membranes is shown in panels II–IV. The antibodies interacted with two bands, approximately 50 and 60 kDa. The size of the 60 kDa polypeptide is consistent with the size of CNGC α in the rod (63 kDa), which is less than expected based on the structure of the channel obtained from cDNA clones. The discrepancy is due to posttranslational cleavage of the N-terminal 92 amino acids [32]. The liver plasma membrane fractions interact with all three antibodies, but with varying intensity, the interaction is strongest with PMc 1D1 (Fig. 4, panel II PM) and the weakest with antibody PMc 2D11 (Fig. 4, panel IV PM). These data indicate that the CNGC α is expressed in the liver to high levels. Preliminary experiments to immunolocalize the channel in liver cells were unsuccessful.

3.5. Ca^{2+} levels in hepatocytes

In order to approximate the relative sensitivity of the channel to different cyclic nucleotides, the effects of cAMP and cGMP on cytosolic free Ca^{2+} levels ($[\text{Ca}^{2+}]_i$) were compared (Fig. 5). Dibutyl cAMP at 10 μM concentration consistently increased $[\text{Ca}^{2+}]_i$ by 20–40% above the baseline. Dibutyl cGMP at this concentration had no effect on $[\text{Ca}^{2+}]_i$ while at 200 μM , at a concentration 20-fold higher than cAMP, dibutyl cGMP increased $[\text{Ca}^{2+}]_i$ also by 20–40%. Thus, cGMP was far less potent than cAMP in eliciting changes in $[\text{Ca}^{2+}]_i$. The effects of sodium nitroprusside, an agent

known to increase cGMP in the liver over 13-fold [33], was also tested. The increase in $[\text{Ca}^{2+}]_i$ with a maximally effective dose was similar to and did not exceed the increases in $[\text{Ca}^{2+}]_i$ observed in Fig. 5. These increases in $[\text{Ca}^{2+}]_i$ upon application of cyclic nucleotides are consistent with the presence of cyclic nucleotide-gated channels in hepatocytes. Mechanisms other than direct gating are conceivable, however, and further studies are needed to establish a precise mechanism for the effect of cyclic nucleotides.

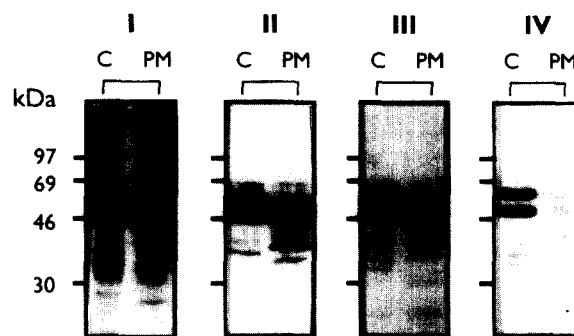


Fig. 3. C-terminal amino acid sequence alignment of rat and human liver CNGC α clones (rows 1 and 2), in comparison with the mouse rod CNGC α sequence. The corresponding sequence of the skeletal muscle is identical to rat liver sequence (not shown). Amino acids are depicted by single letter symbols. The cyclic nucleotide binding domain is shaded. Residues which deviate in the three sequences are shaded.

cyclic nucleotide binding domain																						
1	P	G	D	Y	I	C	K	K	G	D	I	G	R	E	M	Y	I	I	K	E	rat liver	
1	P	G	D	Y	I	C	K	K	G	D	I	G	R	E	M	Y	I	I	K	E	human liver	
493	P	G	D	Y	I	C	K	K	G	D	I	G	R	E	M	Y	I	I	K	E	mouse rod	
21	G	K	L	A	V	V	A	D	D	G	I	T	Q	F	V	V	L	S	D	G	rat liver	
21	G	K	L	A	V	V	A	D	D	G	V	T	Q	F	V	V	L	S	D	G	human liver	
513	G	K	L	A	V	V	A	D	D	G	I	T	Q	F	V	V	L	S	D	G	mouse rod	
41	S	Y	F	G	E	I	S	I	L	N	I	K	G	S	K	A	G	N	R	R	rat liver	
41	S	T	F	G	E	I	S	I	L	N	I	K	G	S	K	A	G	N	R	R	human liver	
533	S	Y	F	G	E	I	S	I	L	N	I	K	G	S	K	A	G	N	R	R	mouse rod	
61	T	A	N	I	K	S	I	G	Y	S	D	L	F	C	L	S	K	D	D	L	rat liver	
61	T	A	N	I	K	S	I	G	Y	S	D	L	F	C	L	S	K	D	D	L	human liver	
553	T	A	N	I	K	S	I	G	Y	S	D	L	F	C	L	S	K	D	D	L	mouse rod	
81	M	E	A	L	T	E	Y	P	D	A	K	T	M	L	E	E	K	G	R	Q	rat liver	
91	M	E	A	L	T	E	Y	P	D	A	K	T	M	L	E	E	K	G	R	Q	human liver	
573	M	E	V	L	T	E	Y	P	D	A	K	T	M	L	E	E	K	G	R	Q	mouse rod	
101	I	L	M	K	D	G	L	L	D	I	N	I	A	N	L	G	S	D	P	K	rat liver	
101	I	L	M	K	D	G	L	L	D	L	N	I	A	N	A	G	S	D	P	K	human liver	
593	I	L	M	K	D	G	L	L	D	I	N	I	A	N	M	G	S	D	P	K	mouse rod	
121	D	L	E	E	K	V	T	R	M	E	G	S	V	D	L	L	Q	T	R	F	rat liver	
121	D	L	E	E	K	V	T	R	M	E	G	S	V	D	L	L	Q	T	R	F	human liver	
613	D	L	E	E	K	V	T	R	M	E	G	S	V	D	L	L	Q	T	R	F	mouse rod	
141	A	R	I	L	A	E	Y	E	S	M	Q	Q	K	L	K	Q	R	L	T	K	rat liver	
141	A	R	I	L	A	E	Y	E	S	M	Q	Q	K	L	K	Q	R	L	T	K	human liver	
633	A	C	I	L	A	E	Y	E	S	M	Q	Q	K	L	K	Q	R	L	T	K	mouse rod	
161	V	E	K	F	L	K	P	L	I	E	T	E	F	S	A	L	E	E	P	G	rat liver	
161	V	E	K	F	L	K	P	L	I	D	T	E	F	S	S	I	E	G	P	W	human liver	
653	V	E	K	F	L	K	P	L	I	E	T	E	F	S	A	L	E	E	P	G	mouse rod	
181	G	E	S	E	P	T	E	S	L	Q	D										201	rat liver
181	S	E	S	G	P	I	D	S	T												188	human liver
673	G	E	S	E	L	T	E	S	L	Q	D										683	mouse rod

Fig. 4. Western blot analysis of hepatic plasma membrane fraction probed with rod CNGC α -specific antibodies. Lanes C: rat retinal homogenates as a positive control; PM: rat hepatic plasma membrane, 100 μ g protein was loaded per lane. Panel I, SDS PAGE stained with Coomassie brilliant blue; II, stained with antibody PMc 1D1 after transfer; III, PMc 1F6; IV, PMc 2G11.

4. Discussion

The results obtained in this study support the notion that CNG channels are present in many non-sensory tissues [15]. According to the data in the Northern blot analysis, the CNGC α subunit and related species are likely to be present in spleen, lung, liver and skeletal muscle. Of these tissues, only the liver and skeletal muscle mRNAs were tested further, employing the PCR technique. Sequence analysis of the PCR product obtained from the rat liver and the skeletal muscle revealed that the amplified portion of the two rat channel subunits are identical with the human rod channel. Since it was the plasma membrane fraction which interacted with the antibodies, while the smooth endoplasmic reticulum derived fraction did not (results not shown), it is likely that in the liver, as in the rod, the channel is present in the plasma membrane [34].

In the rod and olfactory channels, increases in cyclic nucleotide levels lead to the opening of the channels. If the channel protein is present in the hepatic plasma membrane and functions similarly, opening the channel could lead to

the influxes of Na⁺ and Ca²⁺, observed following hormone administration [20,22–24,35,36]. In the measurements of increases in [Ca²⁺], cAMP was far more effective than cGMP in eliciting a response. If increases in cytosolic-free Ca²⁺ levels are proportional to Ca²⁺ influx (which has not been established) then the measurements of the increases in it evoked by the cyclic nucleotides indicate that the hepatic channel is more sensitive to cAMP than to cGMP. The cAMP sensitivity may be conferred by the β subunit, as has been observed in the olfactory system [11]. Further studies are needed to determine the electrophysiological characteristics of the presumably multimeric channel, the influence on cyclic nucleotide specificity of a putative β subunit, and the mechanism involved in its activation/gating of the channel. It is possible that this channel plays a major role in hormonally evoked cation fluxes, including the increases in cytosolic free Ca²⁺ levels. Thus, it could be a link in the chain of events evoked by hormones which act by increasing cyclic nucleotide levels, culminating, -in the case of the liver-, in increased glucose output.

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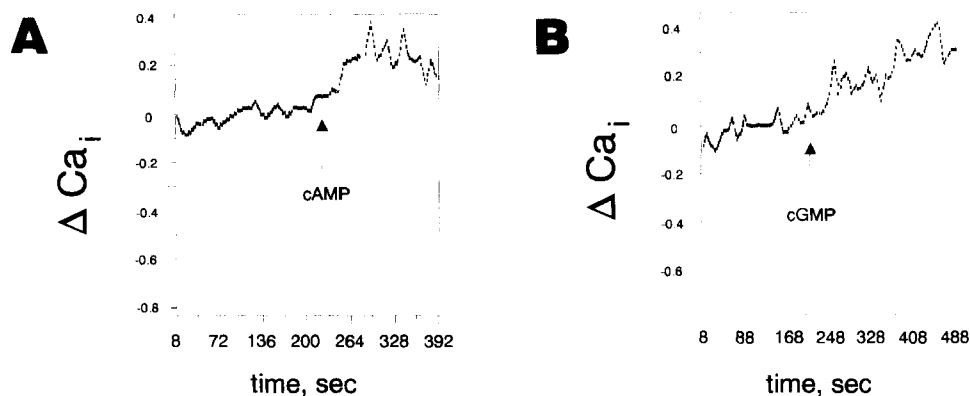


Fig. 5. Measurement of $[Ca^{2+}]_i$ following cyclic nucleotide administration in isolated hepatocytes. $[Ca^{2+}]_i$ was measured as described in Section 2 and normalized as $(F_0 - F_{obs})/F_0$ where F_0 is the starting baseline Ca^{2+} concentration and F_{obs} is the measured Ca^{2+} concentration. As a mean of many experiments, the baseline Ca^{2+} concentration before addition of cyclic nucleotides was 427 ± 72 nM free. At the arrow in A and B, dibutyryl-cAMP (10 μ M) or dibutyryl cGMP (200 μ M), respectively, was added.

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