INDUCTION OF THE ACROSOME REACTION IN GUINEA PIG SPERMATOZOA BY cGMP ANALOGUES

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ABSTRACT

The effect of cyclic nucleotide analogues upon the immediate induction of the guinea pig acrosome reaction (AR) was studied. Dibutyryl (dB) cGMP and 8-bromo-cGMP, when added to sperm suspensions after varying periods of preincubation in glucose-free BWW medium (NaCl 94.59 mM, KCl 4.7 mM, $CaCl_2$ 1.71 mM, KH_2PO_4 1.19 mM, $MgSO_4$ 1.19 mM, $NaHCO_3$ 25.07 mM, pyruvate 0.25 mM, lactate 21.58 mM, and bovine serum albumen 1 g/liter), induced the AR in a large proportion of spermatozoa relative to controls. The proportion of ARs induced upon the addition of dB cGMP or 8-bromo-cGMP (10 mM) at 1 h was equivalent to that obtained after a 5-h incubation in glucose-free BWW alone. The effect of cGMP analogues was concentration dependent over the tested range of 2–12 mM (<1–20%). The simultaneous addition of imidazole (10 mM), a cAMP phosphodiesterase stimulator, potentiated the effect (imidazole + 12 mM 8-bromo-cGMP: 73%). cAMP analogues were without effect. The presence of extracellular Ca^{++} was required, and it is suggested that a rise in the cGMP/cAMP ratio triggers Ca^{++} influx and the AR.

The acrosome reaction (AR) is a massive exocytosis of the acrosome vesicle which occurs in capacitated sperm before fertilization. Early studies claimed that pretreatment of spermatozoa with exogenous cAMP analogues enhanced capacitation as evidenced by increased rates of egg penetration (2, 15, 18). Recently, however, Garcia and Rogers (3, 12) determined that the incubation of guinea pig spermatozoa for 4 h within a capacitating medium, Minimum Culture Medium (MCM), containing dibutyryl (dB) cAMP (10 mM) or phosphodiesterase inhibitors reduced the acrosome reaction percentage as compared to controls (10 vs. 50%). On the other hand, the presence of imidazole, a cAMP phosphodiesterase stimulator, increased the numbers of ARs over controls (90 vs. 50%). The effects of these agents were concentration dependent over the tested range of 1–10 mM. The data reviewed thus far suggest that capacitation may involve a rise in intracellular cAMP levels, but the subsequent AR may be associated with a reduction in cAMP levels.

In accord with this hypothesis are the results of cAMP effects upon other secretory systems. For example, incubation of neutrophils, basophils, and mast cells in media containing agents that promote a rise in cAMP levels inhibits the release of secretory granule contents (for reviews, see references 10 and 11). On the other hand, an increase in cGMP levels promotes the release of secretory granule contents in these same cells. Calcium ion influx is requisite for such secretory activity, and apparently plays a significant role in modulating intracellular levels of cGMP (5). One interpretation of such data is that cGMP and cAMP control opposing cellular function (Yin-Yang hypothesis,

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reference 6). The fact that extracellular Ca^{++} is required for the AR and that its influx parallels or precedes the occurrence of the AR (16, 19) may be related to cyclic nucleotide flux. Indeed, cAMP and cGMP may control opposing cellular functions in sperm as they apparently do in other secretory systems. It may be possible, then, that a rise in the intracellular cGMP/cAMP ratio is associated with the mammalian AR. Accordingly, in this report, the effects of cyclic nucleotide analogues on the AR of guinea pig spermatozoa were investigated.

MATERIALS AND METHODS

Guinea pig epididymal spermatozoa were extruded into 0.9% NaCl in H_2O, disaggregated by pipetting, and washed two times by centrifugation (1,000 *g*, 4 min each). Spermatozoa were then suspended in glucose-free BWW medium (NaCl 94.59 mM, KCl 4.7 mM, $CaCl_2$ 1.71 mM, KH_2PO_4 1.19 mM, $MgSO_4$ 1.19 mM, $NaHCO_3$ 25.07 mM, pyruvate 0.25 mM, lactate 21.58 mM, and bovine serum albumen 1 g/liter) at a final concentration of 1–1.5 $\times 10^7$ spermatozoa/ml and were incubated at 37°C for 5 h in screw-capped tubes under an air atmosphere. Calcium ion-free medium was also employed. Media were prepared daily, immediately before use. The initial pH was adjusted to 7.55, and throughout the experiments it remained <8.00.

At 0 and 1 h after the suspension of spermatozoa, db cAMP, 8-bromo-cAMP (0.001–10 mM), dB cGMP, 8-bromo-cGMP (0.001–10 mM), AMP, 8-bromo-AMP (10 mM), GMP, or bromo-GMP (10 mM) was added to sperm samples. Butyrate (0.1 and 10 mM) was also tested as a control for possible butyrate contamination of the dibutyryl cyclic nucleotides. After a 20-min incubation with these agents, ARs were assessed. In this report, direct counts of acrosome-reacted spermatozoa were made by hemocytometer,[1] six to eight chambers (0.5 mm^3 each) being counted for each condition. ARs were not induced artifactually by the use of the counting apparatus. Counting was rapid and was facilitated because activated, acrosome-reacted spermatozoa tend to remain within confined areas. Care was taken, however, to focus through the 0.1-mm counting chamber depth so as not to overlook cells. Standard errors of the mean averaged <20%. The results are reported as a percentage of AR in the total motile sperm population ($\%AR^{tot}$ = [concn of AR spermatozoa/concn of total spermatozoa − concn of immotile spermatozoa] × 100). Sperm samples were also qualitatively evaluated in wet mounts without coverslips by light and differential interference microscopy. The speed of onset of ARs as well as the incidence of autoagglutination over time was determined. For electron microscopy, spermatozoa were prepared as described previously (7) and examined with a Philips 300 electron microscope at 80 kV.

cAMP levels were determined by the protein binding technique (cAMP Assay Kit, Amersham Corp., Arlington Heights, Ill.) and were corroborated by the antibody technique (cAMP Assay Kit, New England Nuclear, Boston, Mass.). After rapid centrifugation for 30 s, cell pellets were sonicated in 2 ml of absolute ethyl alcohol. After centrifugation, the supernate was evaporated to dryness at 75°C with a stream of air, and the residue was resuspended in buffer for assay. Counts were made with a Beckman LS-250 Liquid Scintillation System (Beckman Instruments, Inc., Fullerton, Calif.) or Nuclear-Chicago gamma counter (Nuclear-Chicago Corp., Des Plaines, Ill.). Recovery of cAMP was ~59%.

RESULTS

Suspension of washed guinea pig spermatozoa in either Ca^{++}-deficient or Ca^{++}-containing, glucose-free BWW resulted in head-to-head agglutination within a matter of minutes (Fig. 1). Incubation in glucose-free BWW from 0 to 5 h (Fig. 2) resulted in a gradual increase in the absolute number of activated, acrosome-reacted spermatozoa, although a large proportion of aggregated, non-acrosome-reacted spermatozoa remained throughout incubation. All agents were without pronounced effect upon AR induction when added at 0 h (Table I). Whereas dB cAMP, 8-bromo-cAMP, AMP, 8-bromo-AMP, GMP, 8-bromo-GMP, and butyrate remained ineffective upon addition at 1 h, dB cGMP or 8-bromo-cGMP (10 mM) was able to induce a large proportion of ARs, being roughly equivalent to the $\%AR^{tot}$ score obtained after a 5-h incubation in glucose-free BWW alone.

Dose-response data were collected to determine the relationship between analogue concentration and $\%AR^{tot}$ scores (Fig. 3). Incremental increases from 2 to 12 mM 8-bromo-cGMP produced increasingly higher numbers of ARs, reaching 20.46% for the highest concentration tested (12 mM). In addition, the simultaneous addition of 10 mM imidazole, a cAMP phosphodiesterase stimulator, caused a dramatic potentiation of the 8-bromo-cGMP effects (Fig. 3). For example, the addition at 1 h of imidazole with 12 mM 8-bromo-cGMP produced a phenomenal 73.94% AR^{tot} score, nearly fourfold greater than 8-bromo-cGMP alone and 112-fold greater than the no-addition

[1] We have chosen not to use the method of Rogers and Yanagimachi (14) to assess the percentage of ARs. Their technique involves placing an aliquot of sperm upon a slide and counting the number of ARs in a sample of 100 motile sperm. The validity of such a sampling technique is directly dependent upon a random distribution of sperm in which each sperm has an equal likelihood of being chosen. Because guinea pig sperm autoagglutinate spontaneously in physiologic media (16, 1) and because these agglutinated sperm, although motile, are non-acrosome reacted, there exists a nonrandom distribution of sperm. Thus, if a sperm is sampled from an aggregate, a priori, it will be unreacted. On the other hand, the chances of sampling an acrosome-reacted sperm from the freely motile pool are very high, because the majority of non-acrosome-reacted sperm are agglutinated. Whenever we have attempted to use this technique, we obtained spuriously high AR scores, with high variability as compared to the hemocytometer technique.

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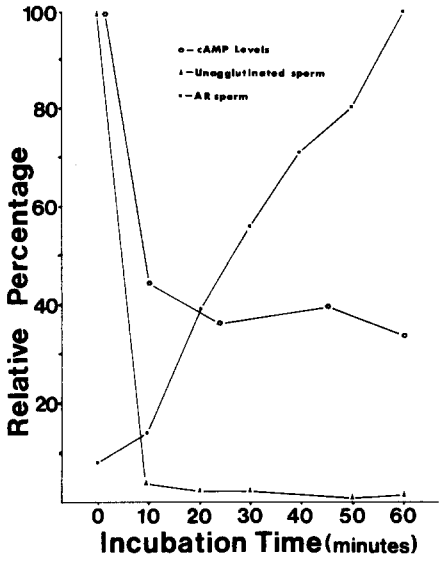

FIGURE 1 The relationship among db cGMP-induced AR, sperm agglutination, and cAMP levels over a 1-h incubation period in glucose-free BWW. Intracellular cAMP levels were assayed 0, 10, 25, 45, and 60 min after suspension of sperm in glucose-free BWW at 37°C. The number of unagglutinated sperm was determined by hemocytometer over the 1-h incubation. Sperm were induced to acrosome react by the addition of 10 mM dB cGMP at 0, 10, 20, 30, 40, 50, or 60 min, and counts were made after a 20-min incubation with the cyclic nucleotide. AR are reported relative to the 60-min score. Sperm concn: 1.5×10^7/ml; cAMP at 0 h: 17 pmol/10^7 sperm; $\%AR^{tot}$ score at 1 h: 12.71.

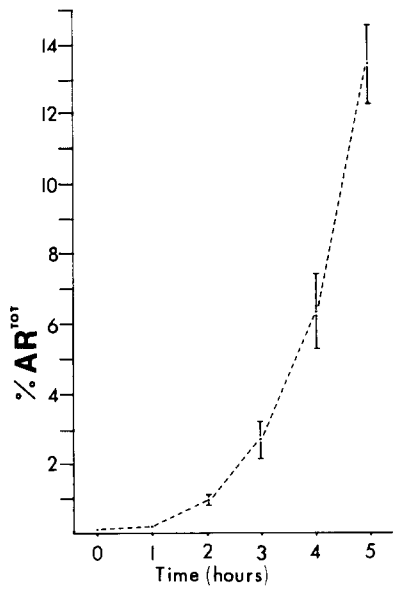

FIGURE 2 The percentage (mean ± SEM) of guinea pig AR occurring during incubation for 5 h in glucose-free BWW.

control. Imidazole alone was ineffective.

The addition of 10 mM dB cGMP but not dB cAMP at 0 h appeared to inhibit agglutination of spermatozoa and did not elicit ARs. If, however, 10 mM dB cGMP was added after agglutination had begun, ARs were induced. This suggested a correlation between agglutination and the ability of spermatozoa to acrosome react. To further evaluate the time dependence of dB cGMP effects, additions of dB cGMP (10 mM) to sperm samples were made at 10-min intervals over a 1-h incubation period and ARs were assessed as described previously (Fig. 1). Results show a time-dependent increase in the ability of dB cGMP to induce ARs. In addition, it was found that, over the 1-h incubation period, sperm cAMP levels decreased from 17 pmol/10^7 sperm to nearly 30% of the initial level and that this decrease was accompanied by a rapid rise in the number of autoagglutinated spermatozoa (Fig. 1).

TABLE I

$\%AR^{tot}$ Score Induced by Addition of Agents after 0- or 1-h Incubation in Glucose-free BWW

	Time	
Addition	0 h	1 h
dB cGMP		
10 mM	0.48 ± 0.14	12.96 ± 1.46
1 mM	0.44 ± 0.07	0.67 ± 0.13
8-bromo-cGMP		
10 mM	0.81 ± 0.20	13.79 ± 1.79
1 mM	0.10 ± 0.02	0.28 ± 0.04
dBc AMP		
10 mM	0.43 ± 0.09	0.95 ± 0.09
1 mM	0.47 ± 0.17	0.58 ± 0.08
8-bromo-cAMP		
10 mM	0.08 ± 0.02	0.28 ± 0.06
1 mM	0.36 ± 0.09	0.23 ± 0.04
AMP		
10 mM	0.05 ± 0.01	0.84 ± 0.06
8-bromo-AMP		
10 mM	0.28 ± 0.07	0.11 ± 0.02
GMP		
10 mM	0.07 ± 0.02	0.95 ± 0.07
8-bromo-GMP		
10 mM	0.02 ± 0.01	0.05 ± 0.01
Butyrate		
10 mM	0.17 ± 0.03	0.69 ± 0.08
0.1 mM	0.11 ± 0.02	0.49 ± 0.11
No addition	0.08 ± 0.03	0.69 ± 0.10

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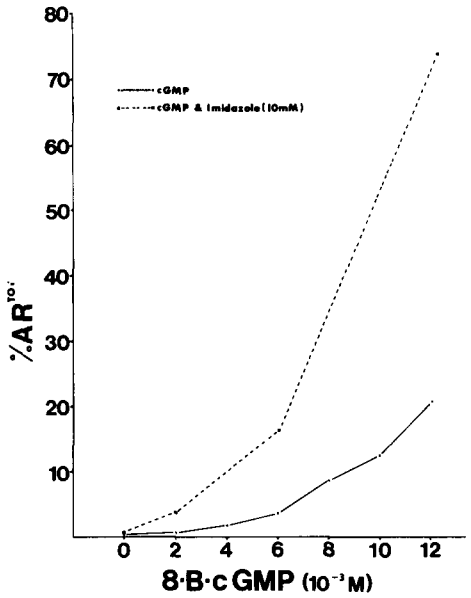

FIGURE 3 Dose-response curves for the induction of ARs by 8-bromo-cGMP or 8-bromo-cGMP + 10 mM imidazole, a cAMP phosphodiesterase stimulator. Sperm were incubated for 1 h at 37°C in glucose-free BWW before addition of drugs. ARs were assessed after an additional 20-min incubation at 37°C in medium containing drugs as indicated.

Typically, within a matter of minutes after the addition of cGMP analogues, vigorous flagellar activity of aggregated spermatozoa ensued. After 5–10 min, freely motile acrosome-reacted spermatozoa were observed attended by an activated pattern of motility (19), and by 15–25 min, a maximum was reached. Activated, acrosome-reacted spermatozoa remained motile for periods up to 2 h, after which an increasingly large number became immotile. ARs were judged physiological as evidenced by differential interference and electron microscopy (Fig. 4).

ARs could not be induced in Ca^{++}-deficient media. In addition, EGTA (2 mM) and nucleotide 5′-triphosphates (ATP, GTP, ITP, UTP; 10 mM) when previously added to Ca^{++}-containing media inhibited AR induction by dB cGMP, and this inhibition could be reversed by a subsequent and sufficient increase in Ca^{++} concentration.

DISCUSSION

A role for cGMP as an effector of the mammalian AR has not been previously considered. Perhaps this is because of the proposal of Gray et al. (9) that guanylate cyclase activity is absent in dog and human spermatozoa. However, their results may have been caused by limitations of their assay procedure, because others (4) have shown that bovine sperm cGMP levels increase more than cAMP levels in the presence of pyruvate and the phosphodiesterase inhibitor, caffeine. Furthermore, cGMP has been localized immunocytochemically within rat spermatids (17). It seems probable that mammalian spermatozoa do possess an active guanylate cyclase.

In a given population, spermatozoa are not in maturational synchrony (8). This is also borne out by the observation that under in vitro conditions known to promote the AR, only a small percentage of the total sperm population will undergo the acrosome reaction at any point in time (Fig. 2). Because sperm nucleotide levels probably fluctuate on an individual basis, the high levels of analogues employed herein were considered necessary to compensate for this individual variability and to produce a large-scale synchronous AR. In addition, there is the possibility of poor membrane permeability of these analogues.

The facts that exogenous cGMP analogues but not cAMP analogues produced an almost immediate AR in a large proportion of spermatozoa and that these spermatozoa were motile for at least 2 h thereafter, lend credence to the idea that an increase, or perhaps a relative increase over cAMP in the levels of intracellular cGMP, promotes the AR. This is further substantiated by the potentiating effect of the cAMP phosphodiesterase stimulator, imidazole. These cyclic nucleotide fluctuations probably initiate Ca^{++} influx, because the absence of Ca^{++} or the presence of agents that chelate calcium ions (EGTA, nucleotide 5′-triphosphates) inhibits the induction of ARs by cGMP analogues. In this regard, the reduction of sperm ATP levels as sperm capacitate (13) may be functionally important, because this reduction may increase the availability of calcium ions, which are requisite for the AR.

The time-dependent effects of dB cGMP upon AR induction indicate that physiologic preparation for the AR, such as capacitation, is occurring. Furthermore, the results reported here suggest that the basis of dB cGMP's increased effectiveness over time may be related to a concomitant decrease in sperm cAMP levels. In effect, then, the cGMP/cAMP ratio increased over time, although the amount of added dB cGMP remained constant at 10 mM. Whether sperm autoagglutination is truly related to the decrease in cAMP levels, as is

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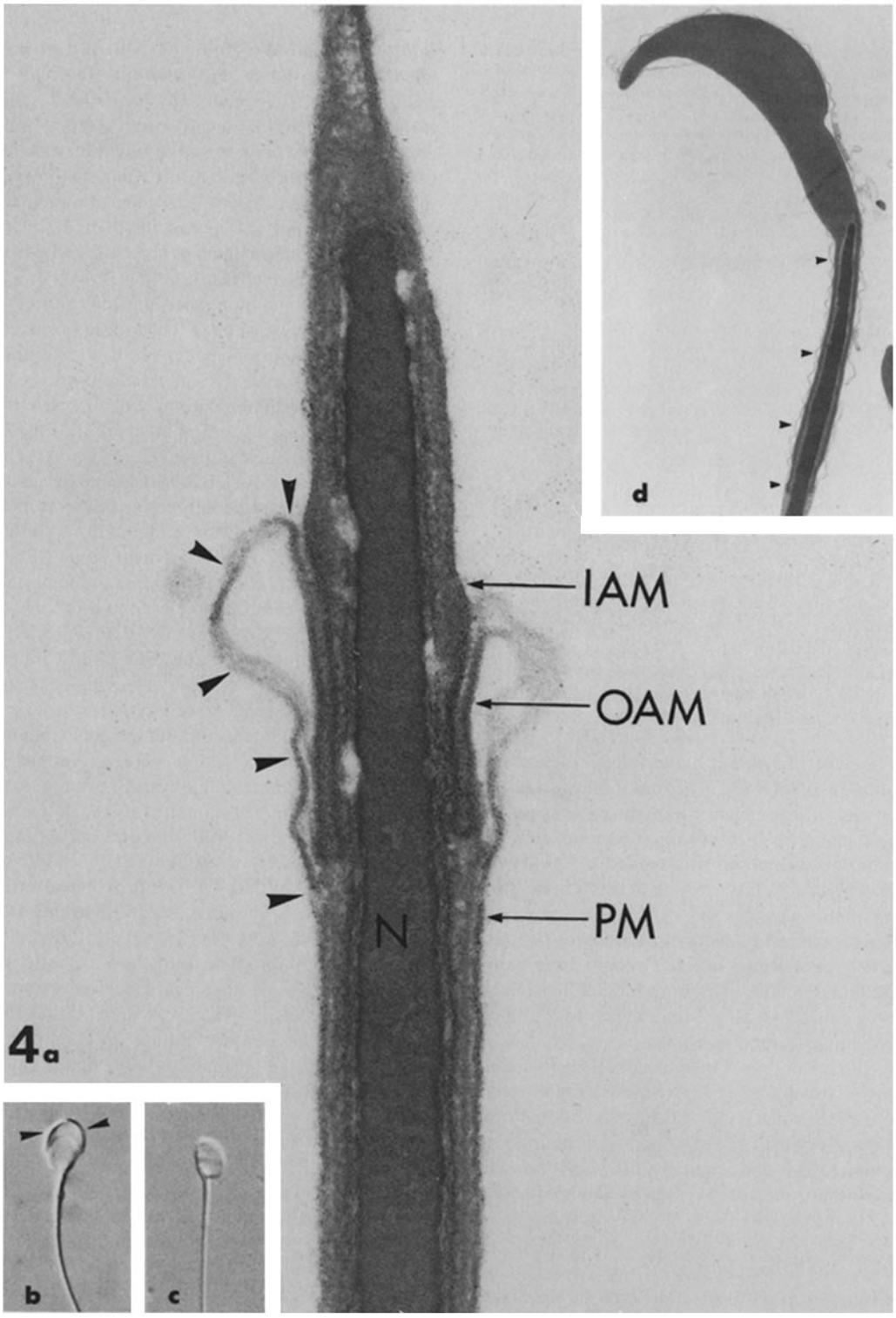

FIGURE 4 (*a*) Electron micrograph of acrosome-reacted guinea pig sperm fixed 20 min after the addition of 10 mM dB cGMP. Note fusion (arrows) of plasmalemma (*PM*) with outer acrosomal membrane (*OAM*) which is typical of physiologcal AR. Inner acrosomal membrane, *IAM*, nucleus, *N*. × 90,000. (*b*) Differential interference micrograph of fixed non-acrosome-reacted sperm. Arrows indicate bulbous acrosome. (*c*) Differential interference micrograph of fixed guinea pig sperm induced to acrosome react by dB cGMP. Note absence of acrosome. (*d*) Electron micrograph of non-acrosome-reacted sperm. Note presence of large acrosome and continuity of plasmalemma (arrows). × 16,000.

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suggested by their inverse correlation, remains to be proven. However, it is plausible that sperm agglutination indicates surface modifications concomitant with capacitation (8).

In sum, the previously reported results that dB cAMP inhibits the AR (3, 12) and those reported here seem to suggest that an increase in the cGMP/cAMP ratio promotes the guinea pig AR.

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